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(21) International Application Number: PCT/CA97/00824 (22) International Filing Date: 3 November 1997 (03.11.97) (30) Priority Data: 60/030,411 4 November 1996 (04.11.96) US (71) Applicant (for all designated States except US): MERCK FROSST CANADA INC. [CA/CA]; 16711 Trans Canada Highway, Kirkland, Quebec H9H 3L1 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): DESMARAIS, Sylvie [CA/CA]; 16711 Trans Canada Highway, Kirkland, Quebec H9H 3L1 (CA). FRIESEN, Richard [CA/CA]; 16711 Trans Canada Highway, Kirkland, Quebec H9H 3L1 (CA). ZAMBONI, Robert [CA/CA]; 16711 Trans Canada Highway, Kirkland, Quebec H9H 3L1 (CA). (74) Agent: MURPHY, Kevin, P.; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College, Montreal, Quebec H3A 2Y3 (CA).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: LIGANDS FOR PHOSPHATASE BINDING ASSAY (57) Abstract Disclosed are new ligands for use in a binding assay for proteases and phosphatases, which contain cysteine in their binding sites or as a necessary structural component for enzymatic binding. The sulfhydryl group of cysteine is the nucleophilic group in the enzyme's mechanistic proteolytic and hydrolytic properties. The assay can be used to determine the ability of new, unknown ligands and mixtures of compounds to competitively bind with the enzyme versus a known binding agent for the enzyme, e.g., a known enzyme inhibitor. By the use of a mutant form of the natural or native wild-type enzyme, in which serine, or another amino acid, e.g., alanine, replaces cysteine, the problem of interference from extraneous oxidizing and alkylating agents in the assay procedure is overcome. The interference arises because of oxidation or alkylation of the sulfhydryl, -SH (or -S ⁻), in the cysteine, which then adversely affects the binding ability of the enzyme. Specifically disclosed is an assay for tyrosine phosphatases and cysteine proteases, including capsases and cathepsins, e.g., Cathepsin K(O ₂), utilizing scintillation proximity assay (SPA) technology. The assay has important applications in the discovery of compounds for the treatment and study of, for example, diabetes, immunosuppression, cancer, Alzheimer's disease and osteoporosis. The novel feature of the use of a mutant enzyme can be extended to its use in a wide variety of conventional colorimetric, photometric, spectrophotometric, radioimmunoassay and ligand-binding competitive assays.		

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TITLE OF THE INVENTION

LIGANDS FOR PHOSPHATASE BINDING ASSAY

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FIELD OF THE INVENTION

This invention relates to the use of mutant phosphatase and protease enzymes in a competitive binding assay. Specific
10 examples are the enzymes, tyrosine phosphatase and cysteine protease, e.g. Cathepsin K, and the assay specifically described is a scintillation proximity assay using a radioactive inhibitor to induce scintillation.

BACKGROUND OF THE INVENTION

15 The use of the scintillation proximity assay (SPA) to study enzyme binding and interactions is a new type of radioimmunoassay and is well known in the art. The advantage of SPA technology over more conventional radioimmunoassay or ligand-binding assays, is that it eliminates the need to separate
20 unbound ligand from bound ligand prior to ligand measurement. See for example, *Nature*, Vol, 341, pp. 167-178 entitled "Scintillation Proximity Assay " by N. Bosworth and P. Towers, *Anal. Biochem.* Vol. 217, pp. 139-147 (1994) entitled "Biotinylated and Cysteine-Modified Peptides as Useful Reagents For Studying the Inhibition of
25 Cathepsin G" by A.M. Brown, et al., *Anal. Biochem.* Vol. 223, pp. 259-265 (1994) entitled "Direct Measurement of the Binding of RAS to Neurofibromin Using Scintillation Proximity Assay" by R. H. Skinner *et al.* and *Anal. Biochem.* Vol. 230, pp. 101-107(1995) entitled "Scintillation Proximity Assay to Measure Binding of Soluble

Fibronectin to Antibody-Captured $\alpha_5\beta_1$ Integrin" by J. A.

Pachter *et al.*

The basic principle of the assay lies in the use of a solid support containing a scintillation agent, wherein a target enzyme is attached to the support through, e.g., a second enzyme-antienzyme linkage. A known tritiated or I^{125} iodinated binding agent, i.e., radioligand inhibitor ligand for the target enzyme is utilized as a control, which when bound to the active site in the target enzyme, is in close proximity to the scintillation agent to induce a scintillation signal, e.g., photon emission, which can be measured by conventional scintillation/radiographic techniques. The unbound tritiated (hot) ligand is too far removed from the scintillation agent to cause an interfering measurable scintillation signal and therefore does not need to be separated, e.g., filtration, as in conventional ligand-binding assays.

The binding of an unknown or potential new ligand (cold, being non-radioactive) can then be determined in a competitive assay versus the known radioligand, by measuring the resulting change in the scintillation signal which will significantly decrease when the unknown ligand also possesses good binding properties.

However, a problem arises when utilizing a target enzyme containing a cysteine group, having a free thiol linkage, -SH, (or present as $-S^-$) which is in the active site region or is closely associated with the active site and is important for enzyme-ligand binding. If the unknown ligand or mixture, e.g. natural product extracts, human body fluids, cellular fluids, etc. contain reagents which can alkylate, oxidize or chemically interfere with the cysteine thiol group such that normal enzyme-ligand binding is disrupted, then false readings will occur in the assay.

What is needed in the art is a method to circumvent and avoid the problem of cysteine interference in the scintillation proximity assay (SPA) procedure in enzyme binding studies.

SUMMARY OF THE INVENTION

We have discovered that by substituting serine for cysteine in a target enzyme, where the cysteine plays an active role in the wild-type enzyme-natural ligand binding process, usually as the catalytic nucleophile in the active binding site, a mutant is formed which can be successfully employed in a scintillation proximity assay without any active site cysteine interference.

This discovery can be utilized for any enzyme which contains cysteine groups important or essential for binding and/or catalytic activity as proteases or hydrolases and includes phosphatases, e.g., tyrosine phosphatases and proteases, e.g. cysteine proteases, including the cathepsins, i.e., Cathepsin K (O2) and the capsases.

Further, use of the mutant enzyme is not limited to the scintillation proximity assay, but can be used in a wide variety of known assays including colorimetric, spectrophotometric, ligand-binding assays, radioimmunoassays and the like.

We have furthermore discovered a new method of amplifying the effect of a binding agent ligand, e.g., radioactive inhibitor, useful in the assay by replacing two or more phosphotyrosine residues with 4-phosphono(difluoromethyl) phenylalanine (F₂Pmp) moieties. The resulting inhibitor exhibits a greater and more hydrolytically stable binding affinity for the target enzyme and a stronger scintillation signal.

By this invention there is provided a process for determining the binding ability of a ligand to a cysteine-containing wild-type enzyme comprising the steps of:

- (a) contacting a complex with the ligand, the complex comprising a mutant form of the wild-type enzyme, in which cysteine, at the active site, is replaced with serine, in the presence of a known binding agent for the mutant enzyme, wherein the binding agent is capable of binding with the mutant enzyme to produce a measurable signal.

Further provided is a process for determining the binding ability of a ligand, preferably a non-radioactive (cold) ligand, to an active site cysteine-containing wild-type tyrosine phosphatase comprising the steps of:

- 5 (a) contacting a complex with the ligand, the complex comprising a mutant form of the wild-type enzyme, the mutant enzyme being PTP1B, containing the same amino acid sequence 1-320 as the wild type enzyme, except at position 215, in which cysteine is replaced with serine in the mutant enzyme, in the presence of a known radioligand binding agent for the mutant enzyme, wherein the binding agent is capable of binding with the mutant enzyme to produce a measurable beta radiation-induced scintillation signal.

Also provided is a new class of peptide binding agents selected from the group consisting of:

- 20 N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide (BzN-EJJ-CONH₂), where E is glutamic acid and J is 4-phosphono(difluoro-methyl)]-L-phenylalanyl;
- N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
- 25 N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
- L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
- L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
- 30 L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
- L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide; and
- L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide; and their tritiated and I¹²⁵ iodinated derivatives.

Further provided is a novel tritiated peptide, tritiated BzN-EJJ-CONH₂, being N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide, wherein E as used herein is glutamic acid and J, as used herein, is the (F₂Pmp) moiety, (4-phosphono(difluoromethyl)-phenylalanyl).

Furthermore there is provided a process for increasing the binding affinity of a ligand for a tyrosine phosphatase or cysteine protease comprising introducing into the ligand two or more 4-phosphono(difluoromethyl)-phenylalanine groups; also provided is the resulting disubstituted ligand.

In addition there is provided a complex comprised of:

- (a) a mutant form of a wild-type enzyme, in which cysteine, necessary for activity in the active site, is replaced with serine and is attached to:
- (b) a solid support.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates the main elements of the invention including the scintillation agent 1, the supporting (fluomicrosphere) bead 5, the surface binding Protein A 10, the linking anti-GST enzyme 15, the fused enzyme construct 20, the GST enzyme 25, the mutant enzyme 30, the tritiated peptide inhibitor 35, the beta radiation emission 40 from the radioactive peptide inhibitor 35 and the emitted light 45 from the induced scintillation.

FIGURE 2 (A and B) illustrates the DNA and amino acid sequences for PTP1B tyrosine phosphatase enzyme, truncated to amino acid positions 1-320. (Active site cysteine at position 215 is in bold and underlined).

FIGURE 3 (A, B and C) illustrates the DNA and amino acid sequences for Cathepsin K. The upper nucleotide sequence represents the cathepsin K cDNA sequence which encodes the cathepsin K preproenzyme (indicated by the corresponding three letter amino acid codes). Numbering indicates the cDNA nucleotide

position. The underlined amino acid is the active site Cys¹³⁹ residue that was mutated to either Ser or Ala.

FIGURE 4 (A and B) illustrates the DNA and amino acid sequences for the capsase, apopain. The upper nucleotide sequence represents the apopain (CPP32) cDNA sequence which encodes the apopain proenzyme (indicated by the corresponding three letter amino acid codes). Numbering indicates the cDNA nucleotide position. The underlined amino acid is the active site Cys¹⁶³ residue that was mutated to Ser.

DETAILED DESCRIPTION OF THE INVENTION

The theory underlying the main embodiment of the invention can be readily seen and understood by reference to FIGURE 1.

Scintillation agent 1 is incorporated into small (yttrium silicate or PVT fluomicro-spheres, AMERSHAM) beads 5 that contain on their surface immunosorbent protein A 10. The protein A coated bead 5 binds the GST fused enzyme construct 20, containing GST enzyme 25 and PTP1B mutant enzyme 30, via anti-GST enzyme antibody 15. When the radioactive e.g., tritiated, peptide 35 is bound to the mutant phosphatase enzyme 30, it is in close enough proximity to the bead 5 for its beta emission 40 (or Auger electron emission in the case of I¹²⁵) to stimulate the scintillation agent 1 to emit light (photon emission) 45. This light 45 is measured as counts in a beta plate counter. When the tritiated peptide 35 is unbound it is too distant from the scintillation agent 1 and the energy is dissipated before reaching the bead 5, resulting in low measured counts. Non-radioactive ligands which compete with the tritiated peptide 35 for the same binding site on the mutant phosphatase enzyme 30 will remove and/or replace the tritiated peptide 35 from the mutant enzyme 30 resulting in lower counts from the uncompetited peptide control. By varying the concentration of the unknown ligand and measuring the resulting lower counts, the inhibition at 50%(IC₅₀) for ligand binding to the mutant enzyme 30 can be obtained. This then is a measure of

the binding ability of the ligand to the mutant enzyme and the wild-type enzyme.

The term "complex" as used herein refers to the assembly containing the mutant enzyme. In its simplest embodiment, the complex is a solid support with the mutant enzyme attached to the surface of the support. A linker can also be employed. As illustrated in FIGURE 1, the complex can further comprise a bead (fluopolymer), anti-enzyme GST/enzyme GST-mutant enzyme-PTP1 linking construct, immunosorbent protein A, and scintillation agent. In general, the complex requires a solid support (beads, immunoassay column of e.g., Al₂O₃, or silica gel) to which the mutant enzyme can be anchored or tethered by attachment through a suitable linker, e.g., an immunosorbent (e.g., Protein A, Protein G, anti-mouse, anti-rabbit, anti-sheep) and a linking assembly, including an enzyme/anti-enzyme construct attached to the solid support.

The term "cysteine-containing wild-type enzyme", as used herein, includes all native or natural enzymes, e.g., phosphatases, cysteine proteases, which contain cysteine in the active site as the active nucleophile, or contain cysteine clearly associated with the active site that is important in binding activity.

The term "binding agent" as used herein includes all ligands (compounds) which are known to be able to bind with the wild-type enzyme and usually act as enzyme inhibitors. The binding agent carries a signal producing agent, e.g., radionuclide, to initiate the measurable signal. In the SPA assay the binding agent is a radioligand.

The term "measurable signal" as used herein includes any type of generated signal, e.g., radioactive, colorimetric, photometric, spectrophotometric, scintillation, which is produced when binding of the radioligand binding agent to the mutant enzyme.

The present invention assay further overcomes problems encountered in the past, where compounds were evaluated by their ability to affect the reaction rate of the enzyme in the phosphatase activity assay. However this did not give direct evidence that compounds were actually binding at the active site of the enzyme. The herein described invention binding assay using a substrate

analog can determine directly whether the mixtures of natural products can irreversibly modify the active site cysteine in the target enzyme resulting in inhibition of the enzymatic activity. To overcome inhibition by these contaminants in the phosphatase assay, a mutated
5 Cys(215) to Ser(215) form of the tyrosine phosphatase PTP1B was cloned and expressed resulting in a catalytically inactive enzyme. In general, replacement of cysteine by serine will lead to a catalytically inactive or substantially reduced activity mutant enzyme.

10 PTP1B is the first protein tyrosine phosphatase to be purified to near homogeneity (Tonks *et al.* *JBC* 263, 6731-6737 (1988)) and sequenced by Charbonneau *et al.* *PNAS* 85, 7182-7186 (1988). The sequence of the enzyme showed substantial homology to a duplicated domain of an abundant protein present in hematopoietic cells
15 variously referred to as LCA or CD45. This protein was shown to possess tyrosine phosphatase activity (Tonks *et al.* *Biochemistry* 27, 8695-8701 (1988)). Protein tyrosine phosphatases have been known to be sensitive to thiol oxidizing agents and alignment of the sequence of PTP1B with subsequently cloned *Drosophila* and mammalian
20 tyrosine phosphatases pointed to the conservation of a Cysteine residue ((M. Strueli *et al.* *Proc. Nat'l Acad USA*, Vol. 86, pp. 8698-7602 (1989)) which when mutated to Ser inactivated the catalytic activity of the enzymes. Guan *et al.* (1991) (*J.B.C.* Vol. 266, 17926-17030, 1991) cloned the rat homologue of PTP1B, expressed a truncated version of
25 the protein in bacteria, purified and showed the Cys at position 215 is the active site residue. Mutation of the Cys²¹⁵ to Ser²¹⁵ resulted in loss of catalytic activity. Human PTP1B was cloned by Chernoff *et al.* *Proc. Natl. Acad. Sci. USA* 87, 2735-2739 (1990).

30 Work leading up to the development of the substrate analog BzN-EJJ-CONH₂ for PTP1B was published by T. Burke *et al.* *Biochem. Biophys. Res. Comm.* 205, pp. 129-134 (1994) with the synthesis of the hexamer peptide containing the phosphotyrosyl mimetic F₂Pmp. We have incorporated the (F₂Pmp) moiety (4-phosphono-(difluoromethyl)phenylalanyl) into various peptides that
35 led to the discovery of BzN-EJJ-CONH₂, (where E is glutamic acid and J as used herein is the F₂Pmp moiety) an active (5 nM) inhibitor

of PTP1B. This was subsequently tritiated giving the radioactive substrate analog required for the binding assay.

5 The mutated enzyme, as the truncated version, containing amino acids 1-320 (see FIGURE 2), has been demonstrated to bind the substrate analog Bz-NEJJ-CONH₂ with high affinity for the first time. The mutated enzyme is less sensitive to oxidizing agents than the wild-type enzyme and provides an opportunity to identify novel inhibitors for this family of enzymes. The use of a mutated enzyme to eliminate interfering contaminants during drug
10 screening is not restricted to the tyrosine phosphatases and can be used for other enzyme binding assays as well.

Other binding assays exist in the art in which the basic principle of this invention can be utilized, namely, using a mutant enzyme in which an important and reactive cysteine important for
15 activity can modified to serine (or a less reactive amino acid) and render the enzyme more stable to cysteine modifying reagents, such as alkylating and oxidizing agents. These other ligand-binding assays include, for example, colorimetric and spectrophotometric assays, e.g. measurement of produced color or fluorescence,
20 phosphorescence (e.g. ELISA, solid absorbant assays) and other radioimmunoassays in which short or long wave light radiation is produced, including ultraviolet and gamma radiation).

Further, the scintillation proximity assay can also be practiced without the fluopolymer support beads (AMERSHAM) as
25 illustrated in FIGURE 1. For example, Scintistrips® are commercially available (Wallac Oy, Finland) and can also be employed as the scintillant-containing solid support for the mutant enzyme complex as well as other solid supports which are conventional in the art.

30 The invention assay described herein is applicable to a variety of cysteine-containing enzymes including protein phosphatases, proteases, lipases, hydrolases, and the like.

The cysteine to serine transformation in the target enzyme can readily be accomplished by analogous use of the
35 molecular cloning technique for Cys²¹⁵ to Ser²¹⁵ described in the below-cited reference by M. Strueli *et al.*, for PTP1B and is hereby incorporated by reference for this particular purpose.

A particularly useful class of phosphatases is the tyrosine phosphatases since they are important in cell function. Examples of this class are: PTP1B, LCA, LAR, DLAR, DPTP(See Strueli et al., below). Ligands discovered by this assay using, for example, PTP1B can be useful, for example, in the treatment of diabetes and immunosuppression.

A useful species is PTP1B, described in *Proc. Nat'l Acad USA*, Vol. 86, pp. 8698-7602 by M. Strueli *et al.* and *Proc. Nat'l Acad Sci. USA*, Vol 87, pp. 2735-2739 by J. Chernoff *et al.*

Another useful class of enzymes is the proteases, including cysteine proteases (thiol proteases), cathepsins and capsases.

The cathepsin class of cysteine proteases is important since Cathepsin K (also termed Cathepsin O2, see *Biol. Chem. Hoppe-Seyler*, Vol. 376 pp. 379-384, June 1995 by D. Bromme *et al.*) is primarily expressed in human osteoclasts and therefore this invention assay is useful in the study and treatment of osteoporosis. See US Patent 5,501,969 (1996) to Human Genome Sciences for the sequence, cloning and isolation of Cathepsin K (O2). See also *J. Biol. Chem.* Vol. 271, No. 21, pp. 12511-12516 (1996) by F. Drake *et al.* and *Biol. Chem. Hoppe-Seyler*, Vol. 376, pp. 379-384(1985) by D. Bromme *et al.*, *supra*.

Examples of the cathepsins include Cathepsin B, Cathepsin G, Cathepsin J, Cathepsin K(O2), Cathesin L, Cathepsin M, Cathepsin S.

The capsase family of cysteine proteases are other examples where the SPA technology and the use of mutated enzymes can be used to determine the ability of unknown compounds and mixtures of compounds to compete with a radioactive inhibitor of the enzyme. An active site mutant of Human Apopain CPP32 (capsase-3) has been prepared. The active site thiol mutated enzymes are less sensitive to oxidizing agents and provide an opportunity to identify novel inhibitors for this family of enzymes.

Examples of the capsase family include: capsase-1(ICE), capsase-2 (ICH-1), capsase-3 (CPP32, human apopain, Yama), capsase-4(ICE_{rel}-11, TX, ICH-2), capsase-5(ICE_{rel}-111, TY), capsase-

6(Mch2), capsase-7(Mch3, ICE-LAP3, CMH-1), capsase-8(FLICE, MACH, Mch5), capsase-9 (ICE-LAP6, Mch6) and capsase-10(Mch4).

Substitution of the cysteine by serine (or by any other amino acid which lowers the activity to oxidizing and alkylating agents, e.g., alanine) does not alter the binding ability of the mutant enzyme to natural ligands. The degree of binding, i.e., binding constant, may be increased or decreased. The catalytic activity of the mutant enzyme will, however, be substantially decreased or even completely eliminated. Thus, natural and synthetic ligands which bind to the natural wild-type enzyme will also bind to the mutant enzyme.

Substitution by serine for cysteine also leads to the mutant enzyme which has the same qualitative binding ability as the natural enzyme but is significantly reduced in catalytic activity. Thus, this invention assay is actually measuring the true binding ability of the test ligand.

The test ligand described herein is a new ligand potentially useful in drug screening purposes and its mode of action is to generally function as an inhibitor for the enzyme.

The binding agent usually is a known ligand used as a control and is capable of binding to the natural wild-type enzyme and the mutant enzyme employed in the assay and is usually chosen as a known peptide inhibitor for the enzyme.

The binding agent also contains a known signal-producing agent to cause or induce the signal in the assay and can be an agent inducing e.g., phosphorescence or fluorescence (ELISA), color reaction or a scintillation signal.

In the instant embodiment, where the assay is a scintillation assay, the signal agent is a radionuclide, i.e., tritium, I^{125} , which induces the scintillant in the solid support to emit measurable light radiation, i.e., photon emission, which can be measured by using conventional scintillation and beta radiation counters.

We have also discovered that introducing two or more 4-phosphonodifluoromethyl phenylalanine (F₂Pmp) groups into a known binding agent greatly enhances the binding affinity of the

binding agent to the enzyme and improves its stability by rendering the resulting complex less susceptible to hydrolytic cleavage.

A method for introducing one F₂Pmp moiety into a ligand is known in the art and is described in detail in *Biochem.*

- 5 *Biophys. Res. Comm.* Vol. 204, pp. 129-134 (1994) hereby incorporated by reference for this particular purpose.

As a result of this technology we discovered a new class of ligands having extremely good binding affinity for PTP1B. These include:

- 10 N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-
15 phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
20 L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide, and
L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide.
- 25 A useful ligand in the series is Bz-NEJJ-CONH₂, whose chemical name is: N-Benzoyl-L-glutamyl-[4-phosphono(difluoro-methyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenyl-alanineamide, and its tritiated form, N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-
30 (difluoromethyl)]-L-phenylalanineamide.

Synthesis of both cold and hot ligands is described in the Examples.

- The following Examples are illustrative of carrying out the invention and should not be construed as being limitations on the
35 scope or spirit of the instant invention.

EXAMPLES

1. Preparation of PTP1B Truncate (Amino Acid Sequence from 1-320
and Fused GST-PTP1B Construct)

An *E. coli* culture carrying a PET plasmid expressing
5 the full length PTP1B protein was disclosed in J. Chernoff *et al. Proc Natl. Acad. Sci. USA*, 87, pp. 2735-2739, (1990). This was modified to a truncated PTP1B enzyme complex containing the active site with amino acids 1-320 inclusive, by the following procedure:

The full length human PTP-1B cDNA sequence
10 (published in J. Chernoff et al., PNAS, USA, *supra*) cloned into a PET vector was obtained from Dr. Raymond Erickson (Harvard University). The PTP-1B cDNA sequence encoding amino acids 1-320 (Seq. ID No. 1) was amplified by PCR using the full length sequence as template. The 5' primer used for the amplification included a
15 Bam HI site at the 5' end and the 3' primer had an Eco RI site at the 3' end. The amplified fragment was cloned into pCR2 (Invitrogen) and sequenced to insure that no sequence errors had been introduced by Taq polymerase during the amplification. This sequence was released from pCR2 by a Bam HI/Eco RI digest and the PTP-1B cDNA
20 fragment ligated into the GST fusion vector pGEX-2T (Pharmacia) that had been digested with the same enzymes. The GST-PTP-1B fusion protein expressed in *E. Coli* has an active protein tyrosine phosphatase activity. This same 1-320 PTP-1B sequence (Seq. ID No. 1) was then cloned into the expression vector pFLAG-2, where FLAG
25 is the octa-peptide AspTyrLysAspAspAspAspLys. This was done by releasing the PTP-1B sequence from the pGEX-2T vector by Nco I/Eco RI digest, filling in the ends of this fragment by Klenow and blunt-end ligating into the blunted Eco RI site of pFLAG2. Site-directed mutagenesis was performed on pFLAG2-PTP-1B plasmid using the
30 Chameleon (Stratagene) double-stranded mutagenesis kit from Stratagene, to replace the active-site Cys-215 with serine. The mutagenesis was carried out essentially as described by the manufacturer and mutants identified by DNA sequencing. The FLAG-PTP-1B Cys215Ser mutant (Seq. ID No. 7) was expressed,
35 purified and found not to have any phosphatase activity. The GST-

PTP-1B Cys²¹⁵Ser mutant was made using the mutated Cys²¹⁵Ser sequence of PTP-1B already cloned into pFLAG2, as follows. The pFLAG2- PTP-1B Cys²¹⁵Ser plasmid (Seq. ID No. 7) was digested with Sal I (3' end of PTP-1B sequence), filled in using Klenow
5 polymerase (New England Biolabs), the enzymes were heat inactivated and the DNA redigested with Bgl II. The 500 bp 3' PTP-1B cDNA fragment which is released and contains the mutated active site was recovered. The pGEX-2T-PTP-1B plasmid was digested with Eco RI (3' end of PTP-1B sequence), filled in by Klenow,
10 phenol/chloroform extracted and ethanol precipitated. This DNA was then digested with Bgl II, producing two DNA fragments a 500 bp 3' PTP-1B cDNA fragment that contains the active site and a 5.5 Kb fragment containing the pGEX-2T vector plus the 5' end of PTP-1B. The 5.5 Kb pGEX-2T 5' PTP-1B fragment was recovered and ligated
15 with the 500 bp Bgl II/Sal I fragment containing the mutated active site. The ligation was transformed into bacteria (type DH5 α , G) and clones containing the mutated active site sequence identified by sequencing. The GST-PTP-1B Cys²¹⁵Ser mutant was overexpressed, purified and found not to have any phosphatase activity.

20

2. Preparation of Tritiated Bz-NEJJ-CONH₂

This compound can be prepared as outlined in Scheme 1, below, and by following the procedures:

25 Synthesis of N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide (BzN-EJJ-CONH₂)

1.0 g of TentaGel® S RAM resin (RAPP polymer, ~ 0.2 mmol/g) as represented by the shaded bead in Scheme 1, was treated
30 with piperidine (3 mL) in DMF (5 mL) for 30 min. The resin (symbolized by the circular P, containing the remainder of the organic molecule except the amino group) was washed successively with DMF (3 x 10 mL) and CH₂Cl₂ (10 mL) and air dried. A solution of DMF (5 mL), N[∞]-Fmoc-4-[diethylphosphono-(difluoromethyl)]-L-

phenylalanine (350 mg) , where Fmoc is 9-fluorenylmethoxycarbonyl, and O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluranium hexafluorophosphate,(acronym being HATU, 228 mg) was treated with diisopropyl-ethylamine (0.21 mL) and, after 15 min., was added to the resin in 3 mL of DMF. After 1 h, the resin was washed successively with DMF (3x10 mL) and CH₂Cl₂ (10 mL) and air dried. The sequence was repeated two times, first using N[∞]-Fmoc-4-[diethylphosphono-(difluoromethyl)]-L-phenylalanine and then using N-Fmoc-L-glutamic acid gamma-*t*-butyl ester. After the final coupling, the resin bound tripeptide was treated with a mixture of piperidine (3 mL) in DMF (5mL) for 30 min. and was then washed successively with DMF (3x10 mL) and CH₂Cl₂ (10 mL) and air dried.

To a solution of benzoic acid (61 mg) and HATU (190 mg) in DMF (1 mL) was added diisopropylethylamine (0.17 mL) and, after 15 min. the mixture was added to a portion of the resin prepared above (290 mg) in 1 mL DMF. After 90 min. the resin was washed successively with DMF (3 x 10 mL) and CH₂Cl₂ (10 mL) and air dried. The resin was treated with 2 mL of a mixture of TFA: water (9:1) and 0.05 mL of triisopropylsilane (TIPS-H) for 1 h. The resin was filtered off and the filtrate was diluted with water (2 mL) and concentrated *in vacuo* at 35°C. The residue was treated with 2.5 mL of a mixture of TFA:DMS:TMSOTf (5:3:1) and 0.05 mL of TIPS-H, and stirred at 25°C for 15 h. (TFA is trifluoroacetic acid, DMS is dimethyl sulfate, TMSOTf is trimethylsilyl trifluoromethanesulfonate).

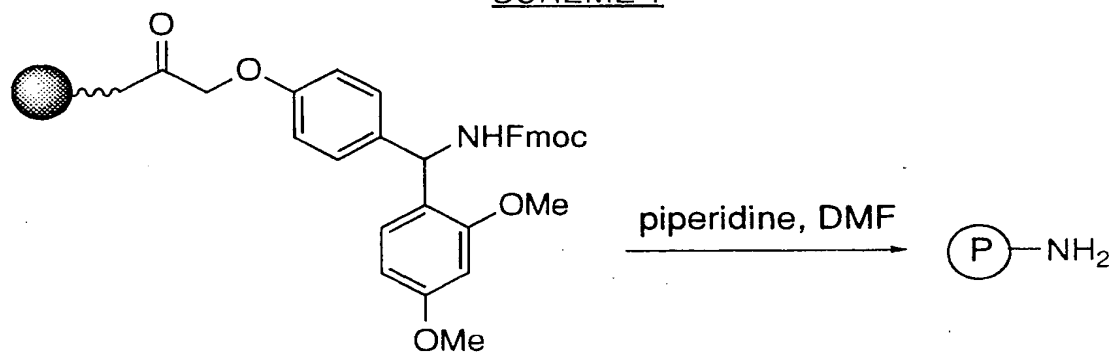
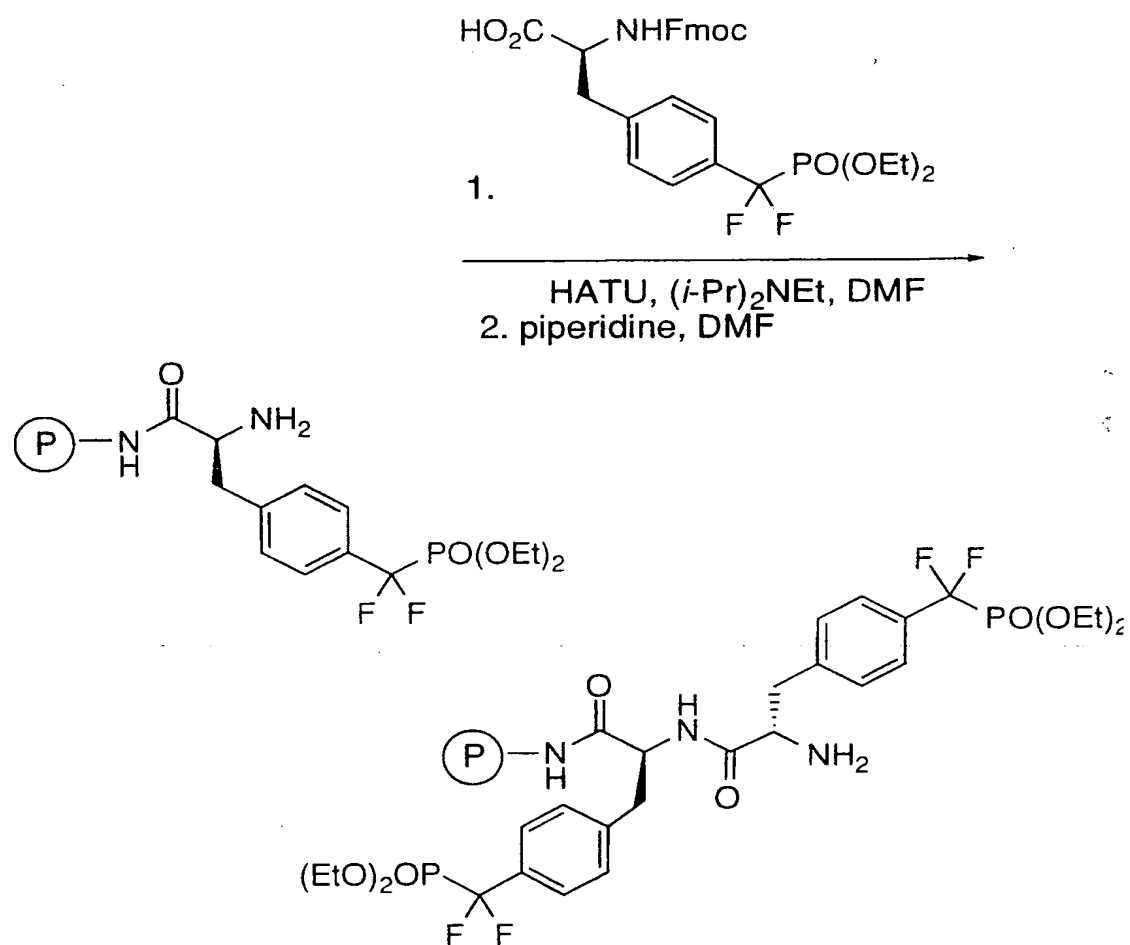
The desired tripeptide, the title compound, was purified by reverse phase HPLC (C18 column, 25 x 100 mm) using a mobile phase gradient from 0.2% TFA in water to 50/50 acetonitrile/0.2% TFA in water over 40 min. and monitoring at 230 nm. The fraction eluting at approximately 14.3 min. was collected, concentrated and lyophilized to yield the title compound as a white foam.

Synthesis of N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide

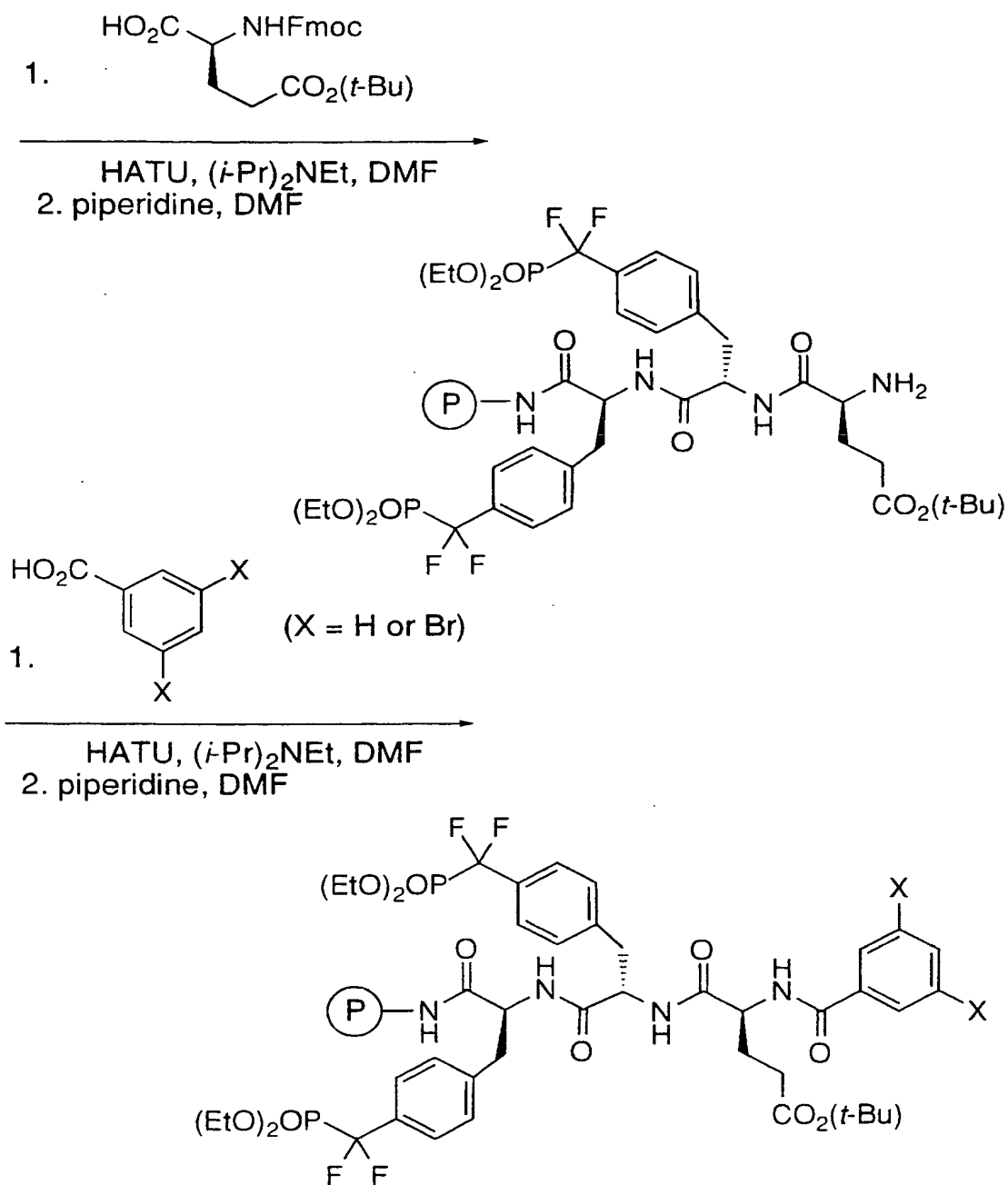
5 The above procedure described for the preparation of BzN-EJJ-CONH₂ was repeated, but substituting 3,5-dibromobenzoic acid for benzoic acid. After HPLC purification as before, except using a gradient over 30 min. and collecting the fraction at approximately 18.3 min., the dibromo containing tripeptide was obtained as a white foam.

10 A portion of this material (2 mg) was dissolved in methanol/triethylamine (0.5 mL, 4/1), 10% Pd-C (2 mg) was added, and the mixture stirred under an atmosphere of tritium gas for 24 h. The mixture was filtered through celite, washing with methanol and the filtrate was concentrated. The title compound was obtained after
15 purification by semi-preparative HPLC using a C18 column and an isocratic mobile phase of acetonitrile/0.2% TFA in water (15:100). The fraction eluting at approximately 5 min. was collected and concentrated *in vacuo*. The title compound was dissolved in 10 mL of methanol/water (9:1) to provide a 0.1 mg/mL solution of specific
20 activity 39.4 Ci/mmol.

SCHEME 1

TentaGel[®] S RAM polymer

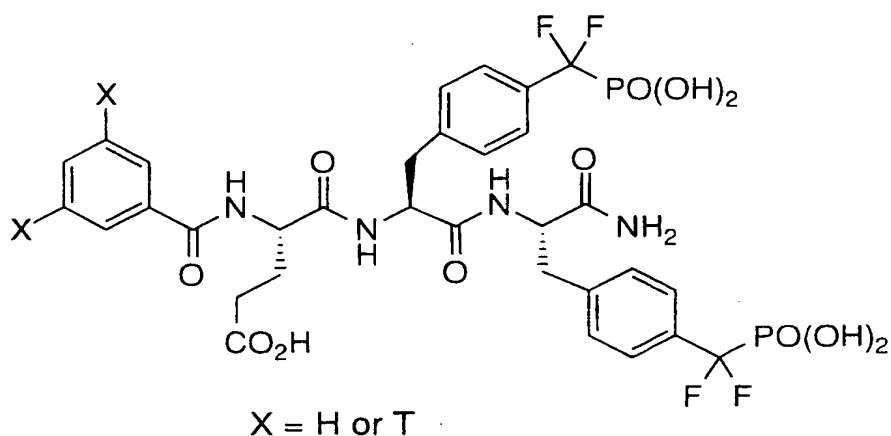
SCHEME 1 CONT'D



SCHEME 1 CONT'D

1. TFA-H₂O (9:1)
2. TFA-DMS-TMSOTf-TIPSH
3. HPLC purification

-
4. for X = Br: T₂ (g), 10% Pd-C
MeOH, Et₃N;
HPLC purification



By following the above described procedure for BzN-EJJ-CONH₂, the following other peptide inhibitors were also similarly prepared:

- 5 N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
- N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
- 10 L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
- L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
- L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
- 15 L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide, and

L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide.

4. Phosphatase Assay Protocol

5

Materials:

EDTA - ethylenediaminetetraacetic acid (Sigma)

DMH - N,N'-dimethyl-N,N'-bis(mercaptoacetyl)-hydrazine (synthesis published in *J. Org. Chem.* 56, pp. 2332-2337, (1991) by R. Singh and G.M. Whitesides and can be substituted with DTT - dithiothreitol Bistris - 2,2-bis(hydroxymethyl)2,2',2''-nitrilotriethanol-(Sigma) Triton X-100 - octylphenolpoly(ethylene-glycolether) 10 (Pierce)

Antibody: Anti-glutathione S-transferase rabbit (H and L) fraction (Molecular Probes)

Enzyme: Human recombinant PTP1B, containing amino acids 1-320, (Seq. ID No. 1) fused to GST enzyme (glutathione S-transferase) purified by affinity chromatography. Wild type (Seq. ID No. 1) contains active site cysteine(215), whereas mutant (Seq. ID No. 7) contains active site serine(215).

Tritiated peptide: Bz-NEJJ-CONH₂, Mwt. 808, empirical formula, C₃₂H₃₂T₂O₁₂P₂F₄

Stock Solutions

25

(10X) Assay Buffer	500 mM Bistris (Sigma), pH 6.2, MW=209.2 20mM EDTA (GIBCO/BRL) Store at 4° C.
--------------------	--

30 Prepare fresh daily:

Assay Buffer (1X) (room temp.)	50 mM Bistris 2 mM EDTA 5 mM DMH (MW=208)
-----------------------------------	---

35

Enzyme Dilution

Buffer (keep on ice) 50 mM Bistris
 2 mM EDTA
 5 mM DMH
5 20% Glycerol (Sigma)
 0.01 mg/ml Triton X-100 (Pierce)

Antibody Dilution

Buffer (keep on ice) 50 mM Bistris
10 2 mM EDTA

IC₅₀ Binding Assay Protocol:

Compounds (ligands) which potentially inhibit the
binding of a radioactive ligand to the specific phosphatase are
15 screened in a 96-well plate format as follows:

To each well is added the following solutions @ 25°C in
the following chronological order:

- 20 1. 110 µl of assay buffer.
2. 10 µl. of 50 nM tritiated BzN-EJJ-CONH₂ in assay
buffer (1X) @ 25°C.
3. 10 µl. of testing compound in DMSO at 10 different
concentrations in serial dilution (final DMSO, about 5% v/v) in
duplicate @ 25°C.
- 25 4. 10 µl. of 3.75 µg/ml purified human recombinant
GST-PTP1B in enzyme dilution buffer.
5. The plate is shaken for 2 minutes.
6. 10 µl. of 0.3 µg/ml anti-glutathione S-transferase
(anti-GST) rabbit IgG (Molecular Probes) diluted in antibody dilution
30 buffer @ 25°C.
7. The plate is shaken for 2 minutes.
8. 50 µl. of protein A-PVT SPA beads (Amersham) @
25°C.
9. The plate is shaken for 5 minutes. The binding
35 signal is quantified on a Microbeta 96-well plate counter.
10. The non-specific signal is defined as the enzyme-
ligand binding in the absence of anti-GST antibody.

11. 100% binding activity is defined as the enzyme-ligand binding in the presence of anti-GST antibody, but in the absence of the testing ligands with the non-specific binding subtracted.
- 5 12. Percentage of inhibition is calculated accordingly.
13. IC₅₀ value is approximated from the non-linear regression fit with the 4-parameter/multiple sites equation (described in: "Robust Statistics", New York, Wiley, by P.J. Huber (1981) and reported in nM units.
- 10 14. Test ligands (compounds) with larger than 90% inhibition at 10 μ M are defined as actives.

The following Table I illustrates typical assay results of examples of known compounds which competitively inhibit the
15 binding of the binding agent, BzN-EJJ-CONH₂.

TABLE I
GST-PTP1B SPA Binding Assay with Non-Mutated (Cys215) and Mutated enzyme (Ser215)

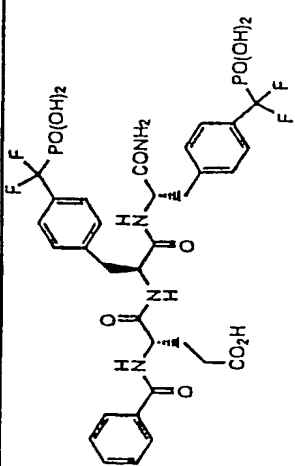
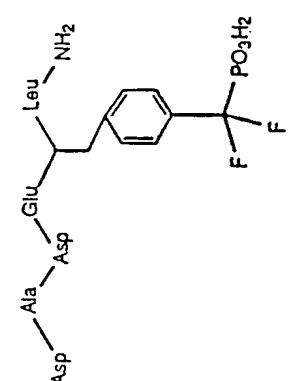
Compound	Structure	Non-Mutated	Mutated
Control:			
Tripeptide(F2PMP)2		14 nM	8 nM
DADP(F2PMP)L hexapeptide (T. Burke et al, Biochem. Biophys. Res. Comm. 204, 129, (1994))		400 nM	100 nM

TABLE I (Cont'd.)

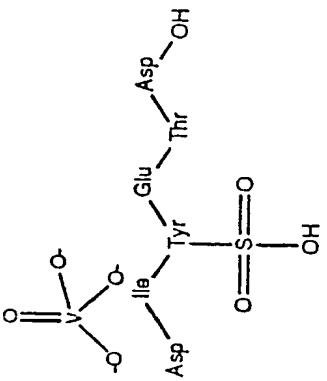
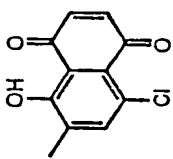
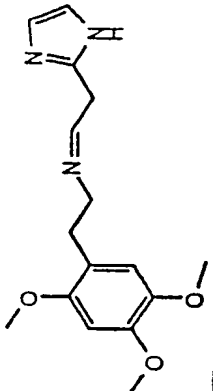
SH-specific binding: Vanadate		2 μ M	>100 μ M
Insulin Receptor Peptide		17 μ M	70 μ M
Potential Oxidizing agents: Hydrogen peroxide	H ₂ O ₂	90% at 83 μ M 4 μ M	0% at 83 μ M >100 μ M
Quinone			
Potential Alkylating agents: Imine		67% at 2 μ M	10% at 2 μ M

TABLE II

Raw Data Counts (dpm)
(duplicates)

no antibody	antibody			conc. BzN-EJJ-CONH2, nM							
(- control)	(+ control)	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0.977	0.488
252	5652	288	873	757	1550	2775	3367	4743	5220	5454	5384
304	6380	273	588	1109	1337	2525	4165	4838	5581	5781	6211

dpm

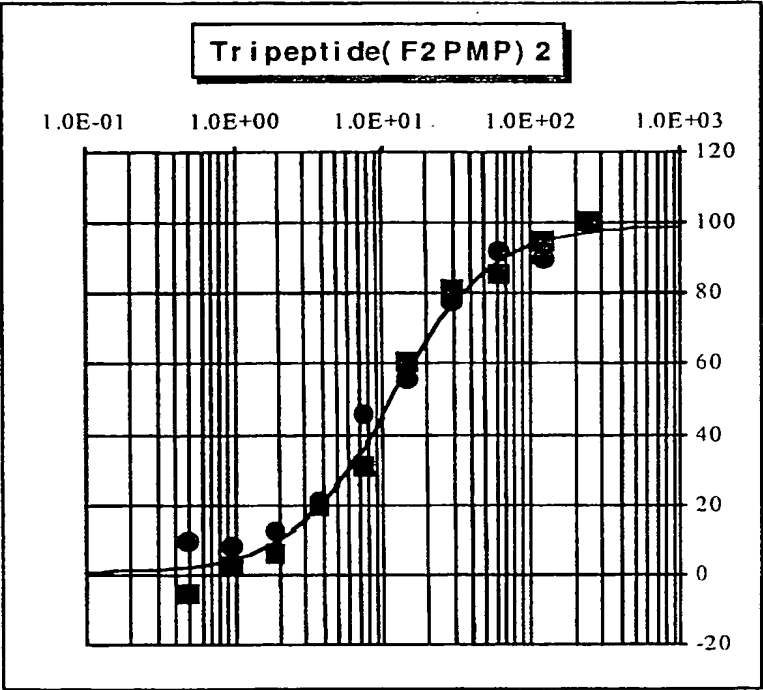
dpm

TABLE III

no antibody	antibody			conc. Bz-EJJ-CONH2, nM							
(- control)	(+ control)	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0.977	0.488
100	5	100	90	92	78	56	45	21	12	8	9
100	-8	100	95	85	81	60	30	19	6	2	-5

% Inh

% Inh



Preparation of Cathepsin K(O2) Mutant (CAT-K Mutant)

Cathepsin K is a prominent cysteine protease in human osteoclasts and is believed to play a key role in osteoclast-mediated bone resorption. Inhibitors of cathepsin K will be useful for the treatment of bone disorders (such as osteoporosis) where excessive bone resorption occurs. Cathepsin K is synthesized as a dormant preproenzyme (Seq. ID No. 4). Both the pre-domain (Met¹-Ala¹⁵) and the prodomain (Leu¹⁶-Arg¹¹⁴) must be removed for full catalytic activity. The mature form of the protease (Ala¹¹⁵-Met³²⁹) contains the active site Cys residue (Cys¹³⁹).

The mature form of cathepsin K is engineered for expression in bacteria and other recombinant systems as a Met Ala¹¹⁵-Met³²⁹ construct by PCR-directed template modification of a clone that is identified. Epitope-tagged variants are also generated: (Met[FLAG]Ala¹¹⁵-Met³²⁹ and Met Ala¹¹⁵-Met³²⁹[FLAG]; where FLAG is the octa-peptide AspTyrLysAspAspAspLys). For the purpose of establishing a binding assay, several other constructs are generated including Met[FLAG]Ala¹¹⁵-[Cys¹³⁹ to Ser¹³⁹]-Met³²⁹ and Met Ala¹¹⁵-[Cys¹³⁹ to Ser¹³⁹]-Met³²⁹[FLAG] (where the active site Cys is mutated to a Ser residue), and Met[FLAG]Ala¹¹⁵-[Cys¹³⁹ to Ala¹³⁹]-Met³²⁹ and Met Ala¹¹⁵-[Cys¹³⁹ to Ala¹³⁹]-Met³²⁹[FLAG] (where the active site Cys is mutated to an Ala residue). In all cases, the resulting re-engineered polypeptides can be used in a binding assay by tethering the mutated enzymes to SPA beads via specific anti-FLAG antibodies that are commercially available (IDI-KODAK). Other epitope tags, GST and other fusions can also be used for this purpose and binding assay formats other than SPA can also be used. Ligands based on the preferred substrate for cathepsin K (e.g. Ac-P2-P1, Ac-P2-P1-aldehydes, Ac-P2-P1-ketones; where P1 is an amino acid with a hydrophilic side chain, preferably Arg or Lys, and P2 is an amino acid with a small hydrophobic side chain, preferably Leu, Val or Phe) are suitable in their radiolabeled (tritiated) forms for SPA-based binding assays. Similar binding assays can also be established for other cathepsin family members.

Preparation of Apopain (caspase-3) Mutant

Apopain is the active form of a cysteine protease belonging to the caspase superfamily of ICE/CED-3 like enzymes. It is derived from a catalytically dormant proenzyme that contains both the 17 kDa large subunit (p17) and 12 kDa (p12) small subunit of the catalytically active enzyme within a 32 kDa proenzyme polypeptide (p32). Apopain is a key mediator in the effector mechanism of apoptotic cell death and modulators of the activity of this enzyme, or structurally-related isoforms, will be useful for the therapeutic treatment of diseases where inappropriate apoptosis is prominent, e.g., Alzheimer's disease.

The method used for production of apopain involves folding of active enzyme from its constituent p17 and p12 subunits which are expressed separately in *E. coli*. The apopain p17 subunit (Ser²⁹-Asp¹⁷⁵) and p12 subunit (Ser¹⁷⁶-His²⁷⁷) are engineered for expression as MetSer²⁹-Asp¹⁷⁵ and MetSer¹⁷⁶-His²⁷⁷ constructs, respectively, by PCR-directed template modification. For the purpose of establishing a binding assay, several other constructs are generated, including a MetSer²⁹-[Cys¹⁶³ to Ser¹⁶³]-Asp¹⁷⁵ large subunit and a Met¹-[Cys¹⁶³ to Ser¹⁶³]-His²⁷⁷ proenzyme. In the former case, the active site Cys residue in the large subunit (p17) is replaced with a Ser residue by site-directed mutagenesis. This large subunit is then re-folded with the recombinant p12 subunit to generate the mature form of the enzyme except with the active site Cys mutated to a Ser. In the latter case, the same Cys¹⁶³ to Ser¹⁶³ mutation is made, except that the entire proenzyme is expressed. In both cases, the resulting re-engineered polypeptides can be used in a binding assay by tethering the mutated enzymes to SPA beads via specific antibodies that are generated to recognize apopain (antibodies against the prodomain, the large p17 subunit, the small p12 subunit and the entire p17:p12 active enzyme have been generated). Epitope tags or GST and other fusions could also be used for this purpose and binding assay formats other than SPA can also be used.

Ligands based on the preferred substrate for apopain (variants of AspGluValAsp), such as Ac- AspGluValAsp, Ac-AspGluValAsp-aldehydes, Ac-AspGluValAsp-ketones are suitable in their radiolabeled forms for SPA-based binding assays. Similar binding
5 assays can also be established for other caspase family members.

DESCRIPTION OF THE SEQUENCE LISTINGS

10 SEQ ID NO. 1 is the top sense DNA strand of Figures 2A and 2B for the PTP1B tyrosine phosphatase enzyme.

SEQ ID NO. 2 is the amino acid sequence of Figures 2A and 2B for the PTP1B tyrosine phosphatase enzyme.

15 SEQ ID NO. 3 is the top sense cDNA strand of Figures 3A, 3B and 3C for the Cathepsin K preproenzyme.

20 SEQ ID NO. 4 is the amino acid sequence of Figures 3A, 3B and 3C for the Cathepsin K preproenzyme.

SEQ ID NO. 5 is the top sense cDNA strand of Figures 4A and 4B for the CPP32 apopain proenzyme.

25 SEQ ID NO. 6 is the amino acid sequence of Figures 4A and 4B for the CPP32 apopain proenzyme.

SEQ ID NO. 7 is the cDNA sequence of the human PTP-1B1-320 Ser mutant.

30 SEQ ID NO. 8 is the amino acid sequence of the human PTP-1B1-320 Ser mutant.

SEQ ID NO. 9 is the cDNA sequence for apopain C163S mutant.

35 SEQ ID NO. 10 is the amino acid sequence for the apopain C163S mutant.

SEQ ID NO. 11 is the large subunit of the heterodimeric amino acid sequence for the apopain C163S mutant.

5 SEQ ID NO. 12 is the cDNA sequence for the Cathepsin K C139S mutant.

SEQ ID NO. 13 is the cDNA sequence for the Cathepsin K C139A mutant.

10 SEQ ID NO. 14 is the amino acid sequence for the Cathepsin K C139S mutant.

15 SEQ ID NO. 15 is the amino acid sequence for the Cathepsin K C139A mutant.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Desmarais, Sylvie
Friesen, Richard
Zamboni, Richard
- (ii) TITLE OF INVENTION: NEW LIGANDS FOR PHOSPHATASE BINDING ASSAY
- (iii) NUMBER OF SEQUENCES: 15
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: ROBERT J. NORTH - MERCK & CO., INC.
 - (B) STREET: 126 EAST LINCOLN AVENUE - P.O. BOX 2000
 - (C) CITY: RAHWAY
 - (D) STATE: NEW JERSEY
 - (E) COUNTRY: USA
 - (F) ZIP: 07065
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US unknown
 - (B) FILING DATE: 04-NOV-1996
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: North, Robert J.
 - (B) REGISTRATION NUMBER: 27,366
 - (C) REFERENCE/DOCKET NUMBER: 19840 PCT
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 732-594-7262
 - (B) TELEFAX: 732-594-4720

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 963 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAAAACCGAA ATAGGTACAG AGACGTCAGT CCCTTTGACC ATAGTCGGAT TAAACTACAT      180
CAAGAAGATA ATGACTATAT CAACGCTAGT TTGATAAAAA TGGAAGAAGC CCAAAGGAGT      240
TACATTCTTA CCCAGGGCCC TTTGCCTAAC ACATGCGGTC ACTTTTGGA GATGGTGTGG      300
GAGCAGAAAA GCAGGGGTGT CGTCATGCTC AACAGAGTGA TGGAGAAAGG TTCGTTAAAA      360
TGCGCACAAAT ACTGGCCACA AAAAGAAGAA AAAGAGATGA TCTTTGAAGA CACAAATTTG      420
AAATTAACAT TGATCTCTGA AGATATCAAG TCATATTATA CAGTGCGACA GCTAGAATTG      480
GAAAACCTTA CAACCCAAGA AACTCGAGAG ATCTTACATT TCCACTATAC CACATGGCCT      540
GACTTTGGAG TCCCTGAATC ACCAGCCTCA TTCTTGAACT TTCTTTTCAA AGTCCGAGAG      600
TCAGGGTCAC TCAGCCCGGA GCACGGGCCC GTTGTGGTGC ACTGCAGTGC AGGCATCGGC      660
AGGTCTGGAA CCTTCTGTCT GGCTGATACC TGCCTCCTGC TGATGGACAA GAGGAAAGAC      720
CCTTCTTCG TTGATATCAA GAAAGTGCTG TTAGAAATGA GGAAGTTTCG GATGGGGTTG      780
ATCCAGACAG CCGACCAGCT GCGCTTCTCC TACCTGGCTG TGATCGAAGG TGCCAAATTC      840
ATCATGGGGG ACTCTTCCGT GCAGGATCAG TGGAAGGAGC TTTCCCACGA GGACCTGGAG      900
CCCCACCCG AGCATATCCC CCCACCTCCC CGGCCACCCA AACGAATCCT GGAGCCACAC      960
TGA                                                                 963

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 320 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Ala Ala Ile Tyr Gln Asp Ile Arg His Glu Ala Ser Asp Phe Pro Cys
 20          25          30
Arg Val Ala Lys Leu Pro Lys Asn Lys Asn Arg Asn Arg Tyr Arg Asp
 35          40          45
Val Ser Pro Phe Asp His Ser Arg Ile Lys Leu His Gln Glu Asp Asn
 50          55          60
Asp Tyr Ile Asn Ala Ser Leu Ile Lys Met Glu Glu Ala Gln Arg Ser
 65          70          75          80
Tyr Ile Leu Thr Gln Gly Pro Leu Pro Asn Thr Cys Gly His Phe Trp
 85          90          95
Glu Met Val Trp Glu Gln Lys Ser Arg Gly Val Val Met Leu Asn Arg
100          105          110

```

Val	Met	Glu	Lys	Gly	Ser	Leu	Lys	Cys	Ala	Gln	Tyr	Trp	Pro	Gln	Lys
		115					120					125			
Glu	Glu	Lys	Glu	Met	Ile	Phe	Glu	Asp	Thr	Asn	Leu	Lys	Leu	Thr	Leu
		130					135				140				
Ile	Ser	Glu	Asp	Ile	Lys	Ser	Tyr	Tyr	Thr	Val	Arg	Gln	Leu	Glu	Leu
145					150					155					160
Glu	Asn	Leu	Thr	Thr	Gln	Glu	Thr	Arg	Glu	Ile	Leu	His	Phe	His	Tyr
				165					170					175	
Thr	Thr	Trp	Pro	Asp	Phe	Gly	Val	Pro	Glu	Ser	Pro	Ala	Ser	Phe	Leu
			180					185					190		
Asn	Phe	Leu	Phe	Lys	Val	Arg	Glu	Ser	Gly	Ser	Leu	Ser	Pro	Glu	His
		195					200					205			
Gly	Pro	Val	Val	Val	His	Cys	Ser	Ala	Gly	Ile	Gly	Arg	Ser	Gly	Thr
		210				215					220				
Phe	Cys	Leu	Ala	Asp	Thr	Cys	Leu	Leu	Leu	Met	Asp	Lys	Arg	Lys	Asp
225				230						235					240
Pro	Ser	Ser	Val	Asp	Ile	Lys	Lys	Val	Leu	Leu	Glu	Met	Arg	Lys	Phe
				245					250					255	
Arg	Met	Gly	Leu	Ile	Gln	Thr	Ala	Asp	Gln	Leu	Arg	Phe	Ser	Tyr	Leu
			260					265					270		
Ala	Val	Ile	Glu	Gly	Ala	Lys	Phe	Ile	Met	Gly	Asp	Ser	Ser	Val	Gln
			275				280					285			
Asp	Gln	Trp	Lys	Glu	Leu	Ser	His	Glu	Asp	Leu	Glu	Pro	Pro	Pro	Glu
		290				295					300				
His	Ile	Pro	Pro	Pro	Pro	Arg	Pro	Pro	Lys	Arg	Ile	Leu	Glu	Pro	His
305					310					315					320

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1669 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAACAAGCA	CTGGATTCCA	TATCCCACTG	CCAAAACCGC	ATGGTTCAGA	TTATCGCTAT	60
TGCAGCTTTC	ATCATAATAC	ACACCTTTGC	TGCCGAAACG	AAGCCAGACA	ACAGATTTCC	120
ATCAGCAGGA	TGTGGGGGCT	CAAGGTTCTG	CTGCTACCTG	TGGTGAGCTT	TGCTCTGTAC	180
CCTGAGGAGA	TACTGGACAC	CCACTGGGAG	CTATGGAAGA	AGACCCACAG	GAAGCAATAT	240
AACAACAAGG	TGGATGAAAT	CTCTCGGCGT	TTAATTTGGG	AAAAAAACCT	GAAGTATATT	300
TCCATCCATA	ACCTTGAGGC	TTCTCTTGGT	GTCCATACAT	ATGAACTGGC	TATGAACCAC	360
CTGGGGGACA	TGACCAGTGA	AGAGGTGGTT	CAGAAGATGA	CTGGACTCAA	AGTACCCCTG	420
TCTCATTCCE	GCAGTAATGA	CACCTTTTAT	ATCCCAGAAT	GGGAAGGTAG	AGCCCCAGAC	480
TCTGTCGACT	ATCGAAAGAA	AGGATATGTT	ACTCCTGTCA	AAAATCAGGG	TCAGTGTGGT	540
TCCTGTTGGG	CTTTTAGCTC	TGTGGGTGCC	CTGGAGGGCC	AACTCAAGAA	GAAACTGGC	600
AAACTCTTAA	ATCTGAGTCC	CCAGAACCTA	GTGGATTGTG	TGTCTGAGAA	TGATGGCTGT	660
GGAGGGGGCT	ACATGACCAA	TGCCTTCCAA	TATGTGCAGA	AGAACCGGGG	TATTGACTCT	720

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GAAGATGCCT ACCCATATGT GGGACAGGAA GAGAGTTGTA TGTACAACCC AACAGGCAAG      780
GCAGCTAAAT GCAGAGGGTA CAGAGAGATC CCCGAGGGGA ATGAGAAAGC CCTGAAGAGG      840
GCAGTGGCCC GAGTGGGACC TGTCTCTGTG GCCATTGATG CAAGCCTGAC CTCCTTCCAG      900
TTTTACAGCA AAGGTGTGTA TTATGATGAA AGCTGCAATA GCGATAATCT GAACCATGCG      960
GTTTTGGCAG TGGGATATGG AATCCAGAAG GGAAACAAGC ACTGGATAAT TAAAAACAGC     1020
TGGGGAGAAA ACTGGGGAAA CAAAGGATAT ATCCTCATGG CTCGAAATAA GAACAACGCC     1080
TGTGGCATTG CCAACCTGGC CAGCTTCCCC AAGATGTGAC TCCAGCCAGC CAAATCCATC     1140
CTGCTCTTCC ATTTCTTCCA CGATGGTGCA GTGTAACGAT GCACTTTGGA AGGGAGTTGG     1200
TGTGCTATTT TTGAAGCAGA TGTGGTGATA CTGAGATTGT CTGTTTCAGTT TCCCCATTTG     1260
TTTGTGCTTC AAATGATCCT TCCTACTTTG CTTCTCTCCA CCCATGACCT TTTTCACTGT     1320
GGCCATCAGG ACTTTCCCTG ACAGCTGTGT ACTCTTAGGC TAAGAGATGT GACTACAGCC     1380
TGCCCCTGAC TGTGTTGTCC CAGGGCTGAT GCTGTACAGG TACAGGCTGG AGATTTTCAC     1440
ATAGGTTAGA TTCTCATTCA CGGGACTAGT TAGCTTTAAG CACCCTAGAG GACTAGGGTA     1500
ATCTGACTTC TCACTTCCTA AGTTCCCTTC TATATCCTCA AGGTAGAAAT GTCTATGTTT     1560
TCTACTCCAA TTCATAAATC TATTCATAAG TCTTTGGTAC AAGTTTACAT GATAAAAAGA     1620
AATGTGATTT GTCTTCCCTT CTTTGCACTT TTGAAATAAA GTATTTATC      1669

```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 329 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Trp Gly Leu Lys Val Leu Leu Leu Pro Val Val Ser Phe Ala Leu
 1          5          10          15
Tyr Pro Glu Glu Ile Leu Asp Thr His Trp Glu Leu Trp Lys Lys Thr
          20          25          30
His Arg Lys Gln Tyr Asn Asn Lys Val Asp Glu Ile Ser Arg Arg Leu
          35          40          45
Ile Trp Glu Lys Asn Leu Lys Tyr Ile Ser Ile His Asn Leu Glu Ala
          50          55          60
Ser Leu Gly Val His Thr Tyr Glu Leu Ala Met Asn His Leu Gly Asp
          65          70          75          80
Met Thr Ser Glu Glu Val Val Gln Lys Met Thr Gly Leu Lys Val Pro
          85          90          95
Leu Ser His Ser Arg Ser Asn Asp Thr Leu Tyr Ile Pro Glu Trp Glu
          100          105          110
Gly Arg Ala Pro Asp Ser Val Asp Tyr Arg Lys Lys Gly Tyr Val Thr
          115          120          125
Pro Val Lys Asn Gln Gly Gln Cys Gly Ser Cys Trp Ala Phe Ser Ser
          130          135          140
Val Gly Ala Leu Glu Gly Gln Leu Lys Lys Lys Thr Gly Lys Leu Leu
          145          150          155          160

```

Asn Leu Ser Pro Gln Asn Leu Val Asp Cys Val Ser Glu Asn Asp Gly
 165 170 175
 Cys Gly Gly Gly Tyr Met Thr Asn Ala Phe Gln Tyr Val Gln Lys Asn
 180 185 190
 Arg Gly Ile Asp Ser Glu Asp Ala Tyr Pro Tyr Val Gly Gln Glu Glu
 195 200 205
 Ser Cys Met Tyr Asn Pro Thr Gly Lys Ala Ala Lys Cys Arg Gly Tyr
 210 215 220
 Arg Glu Ile Pro Glu Gly Asn Glu Lys Ala Leu Lys Arg Ala Val Ala
 225 230 235 240
 Arg Val Gly Pro Val Ser Val Ala Ile Asp Ala Ser Leu Thr Ser Phe
 245 250 255
 Gln Phe Tyr Ser Lys Gly Val Tyr Tyr Asp Glu Ser Cys Asn Ser Asp
 260 265 270
 Asn Leu Asn His Ala Val Leu Ala Val Gly Tyr Gly Ile Gln Lys Gly
 275 280 285
 Asn Lys His Trp Ile Ile Lys Asn Ser Trp Gly Glu Asn Trp Gly Asn
 290 295 300
 Lys Gly Tyr Ile Leu Met Ala Arg Asn Lys Asn Ala Cys Gly Ile
 305 310 315 320
 Ala Asn Leu Ala Ser Phe Pro Lys Met
 325

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1001 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGCAGGAAT TCGGCACGAG GGGTGCTATT GTGAGGCGGT TGTAGAAGTT AATAAAGGTA 60
 TCCATGGAGA ACACTGAAAA CTCAGTGGAT TCAAAATCCA TTAAAAATTT GGAACCAAAG 120
 ATCATACATG GAAGCGAATC AATGGACTCT GGAATATCCC TGGACAACAG TTATAAAATG 180
 GATTATCCTG AGATGGGTTT ATGTATAATA ATTAATAATA AGAATTTTCA TAAGAGCACT 240
 GGAATGACAT CTCGGTCTGG TACAGATGTC GATGCAGCAA ACCTCAGGGA AACATTCAGA 300
 AACTTGAAAT ATGAAGTCAG GAATAAAAAT GATCTTACAC GTGAAGAAAT TGTGGAATTG 360
 ATGCGTGTATG TTTCTAAAGA AGATCACAGC AAAAGGAGCA GTTTTGTGTTG TGTGCTTCTG 420
 AGCCATGGTG AAGAAGGAAT AATTTTTTGA ACAAATGGAC CTGTTGACCT GAAAAAATA 480
 ACAAACCTTTT TCAGAGGGGA TCGTTGTAGA AGTCTAACTG GAAAACCCAA ACTTTTCATT 540
 ATTCAGGCCT GCCGTGGTAC AGAACTGGAC TGTGGCATTG AGACAGACAG TGGTGTGAT 600
 GATGACATGG CGTGTCTATAA AATACCACTG GAGGCCGACT TCTTGATATGC ATACTCCACA 660
 GCACCTGGTT ATTATTCTTG GCGAAATTCA AAGGATGGCT CCTGGTTCAT CCAGTCGCTT 720
 TGTGCCATGC TGAAACAGTA TGCCGACAAG CTTGAATTTA TGCACATTCT TACCCGGGTT 780
 AACCGAAAGG TGGCAACAGA ATTTGAGTCC TTTTCCTTTG ACGCTACTTT TCATGCAAAG 840

AAACAGATTC CATGTATTGT TTCCATGCTC ACAAAGAAGAC TCTATTTTTC TCACTAAAGA 900
 AATGGTTGGT TGGTGGTTTT TTTTAGTTTG TATGCCAAGT GAGAAGATGG TATATTTGGT 960
 ACTGTATTTTC CCTCTCATTG TGACCTACTC TCATGCTGCA G 1001

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 277 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Glu	Asn	Thr	Glu	Asn	Ser	Val	Asp	Ser	Lys	Ser	Ile	Lys	Asn	Leu
1				5					10					15	
Glu	Pro	Lys	Ile	Ile	His	Gly	Ser	Glu	Ser	Met	Asp	Ser	Gly	Ile	Ser
			20					25					30		
Leu	Asp	Asn	Ser	Tyr	Lys	Met	Asp	Tyr	Pro	Glu	Met	Gly	Leu	Cys	Ile
		35					40					45			
Ile	Ile	Asn	Asn	Lys	Asn	Phe	His	Lys	Ser	Thr	Gly	Met	Thr	Ser	Arg
	50					55					60				
Ser	Gly	Thr	Asp	Val	Asp	Ala	Ala	Asn	Leu	Arg	Glu	Thr	Phe	Arg	Asn
65					70					75					80
Leu	Lys	Tyr	Glu	Val	Arg	Asn	Lys	Asn	Asp	Leu	Thr	Arg	Glu	Glu	Ile
				85					90					95	
Val	Glu	Leu	Met	Arg	Asp	Val	Ser	Lys	Glu	Asp	His	Ser	Lys	Arg	Ser
			100					105					110		
Ser	Phe	Val	Cys	Val	Leu	Leu	Ser	His	Gly	Glu	Glu	Gly	Ile	Ile	Phe
		115					120					125			
Gly	Thr	Asn	Gly	Pro	Val	Asp	Leu	Lys	Lys	Ile	Thr	Asn	Phe	Phe	Arg
		130				135					140				
Gly	Asp	Arg	Cys	Arg	Ser	Leu	Thr	Gly	Lys	Pro	Lys	Leu	Phe	Ile	Ile
145					150					155					160
Gln	Ala	Cys	Arg	Gly	Thr	Glu	Leu	Asp	Cys	Gly	Ile	Glu	Thr	Asp	Ser
			165						170					175	
Gly	Val	Asp	Asp	Asp	Met	Ala	Cys	His	Lys	Ile	Pro	Val	Glu	Ala	Asp
			180					185					190		
Phe	Leu	Tyr	Ala	Tyr	Ser	Thr	Ala	Pro	Gly	Tyr	Tyr	Ser	Trp	Arg	Asn
		195					200					205			
Ser	Lys	Asp	Gly	Ser	Trp	Phe	Ile	Gln	Ser	Leu	Cys	Ala	Met	Leu	Lys
	210					215					220				
Gln	Tyr	Ala	Asp	Lys	Leu	Glu	Phe	Met	His	Ile	Leu	Thr	Arg	Val	Asn
225				230						235					240
Arg	Lys	Val	Ala	Thr	Glu	Phe	Glu	Ser	Phe	Ser	Phe	Asp	Ala	Thr	Phe
				245					250					255	
His	Ala	Lys	Lys	Gln	Ile	Pro	Cys	Ile	Val	Ser	Met	Leu	Thr	Lys	Glu
			260					265					270		
Leu	Tyr	Phe	Tyr	His											
			275												

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 963 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

ATGGAGATGG AAAAGGAGTT CGAGCAGATC GACAAGTCCG GGAGCTGGGC GGCCATTTAC      60
CAGGATATCC GACATGAAGC CAGTGACTTC CCATGTAGAG TGGCCAAGCT TCCTAAGAAC      120
AAAAACCGAA ATAGGTACAG AGACGTCAGT CCCTTTGACC ATAGTCGGAT TAAACTACAT      180
CAAGAAGATA ATGACTATAT CAACGCTAGT TTGATAAAAA TGAAGAAGC CCAAAGGAGT      240
TACATTCTTA CCCAGGGCCC TTTGCCTAAC ACATGCGGTC ACTTTTGGGA GATGGTGTGG      300
GAGCAGAAAA GCAGGGGTGT CGTCATGCTC AACAGAGTGA TGGAGAAAGG TTCGTTAAAA      360
TGCGCACAAT ACTGGCCACA AAAAGAAGAA AAAGAGATGA TCTTTGAAGA CACAAATTTG      420
AAATTAACAT TGATCTCTGA AGATATCAAG TCATATTATA CAGTGCGACA GCTAGAATTG      480
GAAAACCTTA CAACCCAAGA AACTCGAGAG ATCTTACATT TCCACTATAC CACATGGCCT      540
GACTTTGGAG TCCCTGAATC ACCAGCCTCA TTCTTGAAC TTTCTTTCAA AGTCCGAGAG      600
TCAGGGTCAC TCAGCCCGGA GCACGGGCCC GTTGTGGTGC ACAGCAGTGC AGGCATCGGC      660
AGGTCTGGAA CCTTCTGTCT GGCTGATACC TGCCTCCTGC TGATGGACAA GAGGAAAGAC      720
CCTTCTTCCG TTGATATCAA GAAAGTGCTG TTAGAAATGA GGAAGTTTCG GATGGGGTTG      780
ATCCAGACAG CCGACCAGCT GCGCTTCTCC TACCTGGCTG TGATCGAAGG TGCCAAATTC      840
ATCATGGGGG ACTCTTCCGT GCAGGATCAG TGGAAGGAGC TTTCCCACGA GGACCTGGAG      900
CCCCACCCG AGCATATCCC CCCACCTCCC CGGCCACCCA AACGAATCCT GGAGCCACAC      960
TGA

```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 322 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Met Glu Met Glu Lys Glu Phe Glu Gln Ile Asp Lys Ser Gly Ser Trp
 1           5           10           15
Ala Ala Ile Tyr Gln Asp Ile Arg His Glu Ala Ser Asp Phe Pro Cys
 20           25           30
Arg Val Ala Lys Leu Pro Lys Asn Lys Asn Arg Asn Arg Tyr Arg Asp
 35           40           45

```

Val	Ser	Pro	Phe	Asp	His	Ser	Arg	Ile	Lys	Leu	His	Gln	Glu	Asp	Asn
50						55					60				
Asp	Tyr	Ile	Asn	Ala	Ser	Leu	Ile	Lys	Met	Glu	Glu	Ala	Gln	Arg	Ser
65					70					75					80
Tyr	Ile	Leu	Thr	Gln	Gly	Pro	Leu	Pro	Asn	Thr	Cys	Gly	His	Phe	Trp
				85					90					95	
Glu	Met	Val	Trp	Glu	Gln	Lys	Ser	Arg	Gly	Val	Val	Met	Leu	Asn	Arg
			100					105					110		
Val	Met	Glu	Lys	Gly	Ser	Leu	Lys	Cys	Ala	Gln	Tyr	Trp	Pro	Gln	Lys
		115					120					125			
Glu	Glu	Lys	Glu	Met	Ile	Phe	Glu	Asp	Thr	Asn	Leu	Lys	Leu	Thr	Leu
	130					135					140				
Ile	Ser	Glu	Asp	Ile	Lys	Ser	Tyr	Tyr	Thr	Val	Arg	Gln	Leu	Glu	Leu
145					150					155					160
Glu	Asn	Leu	Thr	Thr	Gln	Glu	Thr	Arg	Glu	Ile	Leu	His	Phe	His	Tyr
				165					170					175	
Thr	Thr	Trp	Pro	Asp	Phe	Gly	Val	Pro	Glu	Ser	Pro	Ala	Ser	Phe	Leu
			180					185					190		
Asn	Phe	Leu	Phe	Lys	Val	Arg	Glu	Ser	Gly	Ser	Leu	Ser	Pro	Glu	His
		195					200					205			
Gly	Pro	Val	Val	Val	His	Ser	Ser	Ala	Gly	Ile	Gly	Thr	Cys	Gly	Arg
	210					215					220				
Ser	Gly	Thr	Phe	Cys	Leu	Ala	Asp	Thr	Cys	Leu	Leu	Leu	Met	Asp	Lys
225					230					235					240
Arg	Lys	Asp	Pro	Ser	Val	Asp	Ile	Lys	Lys	Val	Leu	Leu	Glu	Met	
				245				250						255	
Arg	Lys	Phe	Arg	Met	Gly	Leu	Ile	Gln	Thr	Ala	Asp	Gln	Leu	Arg	Phe
			260					265					270		
Ser	Tyr	Leu	Ala	Val	Ile	Glu	Gly	Ala	Lys	Phe	Ile	Met	Gly	Asp	Ser
		275					280					285			
Ser	Val	Gln	Asp	Gln	Trp	Lys	Glu	Leu	Ser	His	Glu	Asp	Leu	Glu	Pro
	290					295					300				
Pro	Pro	Glu	His	Ile	Pro	Pro	Pro	Pro	Arg	Pro	Pro	Lys	Arg	Ile	Leu
305					310					315					320
Glu	Pro														

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1001 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCAGGAAT TCGGCACGAG GGGTGCTATT GTGAGGCGGT TGTAGAAGTT AATAAAGGTA	60
TCCATGGAGA ACACTGAAAA CTCAGTGGAT TCAAAATCCA TTAAAAATTT GGAACCAAAG	120
ATCATACATG GAAGCGAATC AATGGACTCT GGAATATCCC TGGACAACAG TTATAAAATG	180
GATTATCCTG AGATGGGTTT ATGTATAATA ATTAATAATA AGAATTTTCA TAAGAGCACT	240
GGAATGACAT CTCGGTCTGG TACAGATGTC GATGCAGCAA ACCTCAGGGA AACATTCAGA	300
AACTTGAAAT ATGAAGTCAG GAATAAAAAT GATCTTACAC GTGAAGAAAT TGTGGAATTG	360


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ATGCGTGATG TTTCTAAAGA AGATCACAGC AAAAGGAGCA GTTTTGTGTTG TGTGCTTCTG      420
AGCCATGGTG AAGAAGGAAT AATTTTTTGGG ACAAATGGAC CTGTTGACCT GAAAAAATA      480
ACAAACTTTT TCAGAGGGGA TCGTTGTAGA AGTCTAACTG GAAAACCCAA ACTTTTCATT      540
ATTGAGGCCT CCCGTGGTAC AGAACTGGAC TGTGGCATTG AGACAGACAG TGGTGTTGAT      600
GATGACATGG CGTGTCATAA AATACCAGTG GAGGCCGACT TCTTGTATGC ATACTCCACA      660
GCACCTGGTT ATTATTCTTG GCGAAATTCA AAGGATGGCT CCTGGTTCAT CCAGTCGCTT      720
TGTGCCATGC TGAAACAGTA TGCCGACAAG CTTGAATTTA TGCACATTCT TACCCGGGTT      780
AACCGAAAGG TGGCAACAGA ATTTGAGTCC TTTTCCTTTG ACGCTACTTT TCATGCAAAG      840
AAACAGATTC CATGTATTGT TTCCATGCTC ACAAAGAAG TCTATTTTGA TCACTAAAGA      900
AATGGTTGGT TGGTGGTTTT TTTTAGTTTG TATGCCAAGT GAGAAGATGG TATATTTGGT      960
ACTGTATTTT CCTCTCATTT TGACCTACTC TCATGCTGCA G                                1001

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(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 277 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

Met Glu Asn Thr Glu Asn Ser Val Asp Ser Lys Ser Ile Lys Asn Leu
 1          5          10          15
Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Asp Ser Gly Ile Ser
 20          25          30
Leu Asp Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile
 35          40          45
Ile Ile Asn Asn Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg
 50          55          60
Ser Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn
 65          70          75          80
Leu Lys Tyr Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile
 85          90          95
Val Glu Leu Met Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser
100          105          110
Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe
115          120          125
Gly Thr Asn Gly Pro Val Asp Leu Lys Lys Ile Thr Asn Phe Phe Arg
130          135          140
Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile
145          150          155          160
Gln Ala Ser Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp Ser
165          170          175
Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro Val Glu Ala Asp
180          185          190
Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp Arg Asn
195          200          205
Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
210          215          220

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Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn
225                230                235                240
Arg Lys Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe
                245                250                255
His Ala Lys Lys Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu
                260                265                270
Leu Tyr Phe Tyr His
                275

```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 277 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Glu Asn Thr Glu Asn Ser Val Asp Ser Lys Ser Ile Lys Asn Leu
 1          5          10          15
Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Asp Ser Gly Ile Ser
 20          25          30
Leu Asp Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile
 35          40          45
Ile Ile Asn Asn Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg
 50          55          60
Ser Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn
 65          70          75          80
Leu Lys Tyr Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile
 85          90          95
Val Glu Leu Met Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser
100          105          110
Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe
115          120          125
Gly Thr Asn Gly Pro Val Asp Leu Lys Lys Ile Thr Asn Phe Phe Arg
130          135          140
Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile
145          150          155          160
Gln Ala Ser Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp Ser
165          170          175
Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro Val Glu Ala Asp
180          185          190
Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp Arg Asn
195          200          205
Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
210          215          220
Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn
225          230          235          240
Arg Lys Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe
245          250          255
His Ala Lys Lys Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu
260          265          270
Leu Tyr Phe Tyr His
275

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 990 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGTGGGGGC	TCAAGGTTCT	GCTGCTACCT	GTGGTGAGCT	TTGCTCTGTA	CCCTGAGGAG	60
ATACTGGACA	CCCACTGGGA	GCTATGGAAG	AAGACCCACA	GGAAGCAATA	TAACAACAAG	120
GTGGATGAAA	TCTCTCGGCG	TTTAATTTGG	GAAAAAACC	TGAAGTATAT	TTCCATCCAT	180
AACCTTGAGG	CTTCTCTTGG	TGTCCATACA	TATGAAGTGG	CTATGAACCA	CCTGGGGGAC	240
ATGACCAGTG	AAGAGGTGGT	TCAGAAGATG	ACTGGACTCA	AAGTACCCCT	GTCTCATTC	300
CGCAGTAATG	ACACCCTTTA	TATCCCAGAA	TGGGAAGGTA	GAGCCCCAGA	CTCTGTGCG	360
TATCGAAAGA	AAGGATATGT	TACTCCTGTC	AAAAATCAGG	GTCAGTGTGG	TTCTCTTGG	420
GCTTTTAGCT	CTGTGGGTGC	CCTGGAGGGC	CAACTCAAGA	AGAAAACTGG	CAAACTCTTA	480
AATCTGAGTC	CCCAGAACCT	AGTGGATTGT	GTGTCTGAGA	ATGATGGCTG	TGGAGGGGGC	540
TACATGACCA	ATGCCTTCCA	ATATGTGCAG	AAGAACCGGG	GTATTGACTC	TGAAGATGCC	600
TACCCATATG	TGGGACAGGA	AGAGAGTTGT	ATGTACAACC	CAACAGGCAA	GGCAGCTAAA	660
TGCAGAGGGT	ACAGAGAGAT	CCCCGAGGGG	AATGAGAAAG	CCCTGAAGAG	GGCAGTGGCC	720
CGAGTGGGAC	CTGTCTCTGT	GGCCATTGAT	GCAAGCCTGA	CCTCCTTCCA	GTTTTACAGC	780
AAAGGTGTGT	ATTATGATGA	AAGCTGCAAT	AGCGATAATC	TGAACCATGC	GGTTTTGGCA	840
GTGGGATATG	GAATCCAGAA	GGGAAACAAG	CACTGGATAA	TTAAAAACAG	CTGGGGAGAA	900
AACTGGGGAA	ACAAAGGATA	TATCCTCATG	GCTCGAAATA	AGAACAACGC	CTGTGGCATT	960
GCCAACCTGG	CCAGCTTCCC	CAAGATGTGA				990

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 990 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATGTGGGGGC	TCAAGGTTCT	GCTGCTACCT	GTGGTGAGCT	TTGCTCTGTA	CCCTGAGGAG	60
ATACTGGACA	CCCACTGGGA	GCTATGGAAG	AAGACCCACA	GGAAGCAATA	TAACAACAAG	120
GTGGATGAAA	TCTCTCGGCG	TTTAATTTGG	GAAAAAACC	TGAAGTATAT	TTCCATCCAT	180

```

AACCTTGAGG CTTCTCTTGG TGTCCATACA TATGAACTGG CTATGAACCA CCTGGGGGAC      240
ATGACCAGTG AAGAGGTGGT TCAGAAGATG ACTGGACTCA AAGTACCCCT GTCTCATTCC      300
CGCAGTAATG ACACCCTTTA TATCCAGAA TGGGAAGGTA GAGCCCCAGA CTCTGTGCGAC      360
TATCGAAAAGA AAGGATATGT TACTCCTGTC AAAAATCAGG GTCAGTGTGG TTCCGCTTGG      420
GCTTTTAGCT CTGTGGGTGC CCTGGAGGGC CAACTCAAGA AGAAAACTGG CAAACTCTTA      480
AATCTGAGTC CCCAGAACCT AGTGGATTGT GTGTCTGAGA ATGATGGCTG TGGAGGGGGC      540
TACATGACCA ATGCCTTCCA ATATGTGCAG AAGAACCGGG GTATTGACTC TGAAGATGCC      600
TACCCATATG TGGGACAGGA AGAGAGTTGT ATGTACAACC CAACAGGCAA GGCAGCTAAA      660
TGCAGAGGGT ACAGAGAGAT CCCCAGGGG AATGAGAAAG CCCTGAAGAG GGCAGTGGCC      720
CGAGTGGGAC CTGTCTCTGT GGCCATTGAT GCAAGCCTGA CCTCCTTCCA GTTTTACAGC      780
AAAGGTGTGT ATTATGATGA AAGCTGCAAT AGCGATAATC TGAACCATGC GGTTTTGGCA      840
GTGGGATATG GAATCCAGAA GGGAAACAAG CACTGGATAA TAAAAACAG CTGGGGAGAA      900
AACTGGGGAA ACAAAGGATA TATCCTCATG GCTCGAAATA AGAACAACGC CTGTGGCATT      960
GCCAACCTGG CCAGCTTCCC CAAGATGTGA

```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 329 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

Met Trp Gly Leu Lys Val Leu Leu Leu Pro Val Val Ser Phe Ala Leu
 1           5           10           15
Tyr Pro Glu Glu Ile Leu Asp Thr His Trp Glu Leu Trp Lys Lys Thr
          20           25           30
His Arg Lys Gln Tyr Asn Asn Lys Val Asp Glu Ile Ser Arg Arg Leu
          35           40           45
Ile Trp Glu Lys Asn Leu Lys Tyr Ile Ser Ile His Asn Leu Glu Ala
          50           55           60
Ser Leu Gly Val His Thr Tyr Glu Leu Ala Met Asn His Leu Gly Asp
          65           70           75           80
Met Thr Ser Glu Glu Val Val Gln Lys Met Thr Gly Leu Lys Val Pro
          85           90           95
Leu Ser His Ser Arg Ser Asn Asp Thr Leu Tyr Ile Pro Glu Trp Glu
          100          105          110
Gly Arg Ala Pro Asp Ser Val Asp Tyr Arg Lys Lys Gly Tyr Val Thr
          115          120          125
Pro Val Lys Asn Gln Gly Gln Cys Gly Ser Ser Trp Ala Phe Ser Ser
          130          135          140
Val Gly Ala Leu Glu Gly Gln Leu Lys Lys Lys Thr Gly Lys Leu Leu
          145          150          155          160
Asn Leu Ser Pro Gln Asn Leu Val Asp Cys Val Ser Glu Asn Asp Gly
          165          170          175

```

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Cys Gly Gly Gly Tyr Met Thr Asn Ala Phe Gln Tyr Val Gln Lys Asn
      180      185      190
Arg Gly Ile Asp Ser Glu Asp Ala Tyr Pro Tyr Val Gly Gln Glu Glu
      195      200      205
Ser Cys Met Tyr Asn Pro Thr Gly Lys Ala Ala Lys Cys Arg Gly Tyr
      210      215      220
Arg Glu Ile Pro Glu Gly Asn Glu Lys Ala Leu Lys Arg Ala Val Ala
      225      230      235      240
Arg Val Gly Pro Val Ser Val Ala Ile Asp Ala Ser Leu Thr Ser Phe
      245      250      255
Gln Phe Tyr Ser Lys Gly Val Tyr Tyr Asp Glu Ser Cys Asn Ser Asp
      260      265      270
Asn Leu Asn His Ala Val Leu Ala Val Gly Tyr Gly Ile Gln Lys Gly
      275      280      285
Asn Lys His Trp Ile Ile Lys Asn Ser Trp Gly Glu Asn Trp Gly Asn
      290      295      300
Lys Gly Tyr Ile Leu Met Ala Arg Asn Lys Asn Asn Ala Cys Gly Ile
      305      310      315      320
Ala Asn Leu Ala Ser Phe Pro Lys Met
      325

```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 329 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```

Met Trp Gly Leu Lys Val Leu Leu Leu Pro Val Val Ser Phe Ala Leu
  1      5      10      15
Tyr Pro Glu Glu Ile Leu Asp Thr His Trp Glu Leu Trp Lys Lys Thr
      20      25      30
His Arg Lys Gln Tyr Asn Asn Lys Val Asp Glu Ile Ser Arg Arg Leu
      35      40      45
Ile Trp Glu Lys Asn Leu Lys Tyr Ile Ser Ile His Asn Leu Glu Ala
      50      55      60
Ser Leu Gly Val His Thr Tyr Glu Leu Ala Met Asn His Leu Gly Asp
      65      70      75      80
Met Thr Ser Glu Glu Val Val Gln Lys Met Thr Gly Leu Lys Val Pro
      85      90      95
Leu Ser His Ser Arg Ser Asn Asp Thr Leu Tyr Ile Pro Glu Trp Glu
      100      105      110
Gly Arg Ala Pro Asp Ser Val Asp Tyr Arg Lys Lys Gly Tyr Val Thr
      115      120      125
Pro Val Lys Asn Gln Gly Gln Cys Gly Ser Ala Trp Ala Phe Ser Ser
      130      135      140
Val Gly Ala Leu Glu Gly Gln Leu Lys Lys Lys Thr Gly Lys Leu Leu
      145      150      155      160
Asn Leu Ser Pro Gln Asn Leu Val Asp Cys Val Ser Glu Asn Asp Gly
      165      170      175
Cys Gly Gly Gly Tyr Met Thr Asn Ala Phe Gln Tyr Val Gln Lys Asn
      180      185      190
Arg Gly Ile Asp Ser Glu Asp Ala Tyr Pro Tyr Val Gly Gln Glu Glu
      195      200      205

```

Ser	Cys	Met	Tyr	Asn	Pro	Thr	Gly	Lys	Ala	Ala	Lys	Cys	Arg	Gly	Tyr
210						215					220				
Arg	Glu	Ile	Pro	Glu	Gly	Asn	Glu	Lys	Ala	Leu	Lys	Arg	Ala	Val	Ala
225					230					235					240
Arg	Val	Gly	Pro	Val	Ser	Val	Ala	Ile	Asp	Ala	Ser	Leu	Thr	Ser	Phe
				245					250					255	
Gln	Phe	Tyr	Ser	Lys	Gly	Val	Tyr	Tyr	Asp	Glu	Ser	Cys	Asn	Ser	Asp
			260					265					270		
Asn	Leu	Asn	His	Ala	Val	Leu	Ala	Val	Gly	Tyr	Gly	Ile	Gln	Lys	Gly
		275					280					285			
Asn	Lys	His	Trp	Ile	Ile	Lys	Asn	Ser	Trp	Gly	Glu	Asn	Trp	Gly	Asn
	290					295					300				
Lys	Gly	Tyr	Ile	Leu	Met	Ala	Arg	Asn	Lys	Asn	Asn	Ala	Cys	Gly	Ile
305					310					315					320
Ala	Asn	Leu	Ala	Ser	Phe	Pro	Lys	Met							
				325											

WHAT IS CLAIMED:

1. A peptide comprising a ligand having binding affinity for a tyrosine phosphatase or cysteine protease, wherein said ligand contains two or more 4-phosphono(difluoromethyl) phenylalanine groups.
2. The peptide of Claim 1 wherein said ligand has a greater binding affinity than the corresponding ligand only containing one of said 4-phosphono(difluoromethyl) phenylalanine groups.
3. A peptide selected from the group consisting of:
N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide (BzN-EJJ-CONH₂), where E is glutamic acid and J is 4-phosphono(difluoro-methyl)]-L-phenylalanyl;
N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono--(difluoromethyl)]-L-phenylalanine amide;
L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide; and
L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide.
4. The peptide of Claim 3 in tritiated or I¹²⁵ iodinated form.
5. A tritiated peptide, N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide.

6. A process for increasing the binding affinity of a ligand for a tyrosine phosphatase or cysteine protease comprising introducing into the ligand two or more 4-phosphono(difluoromethyl) phenylalanine groups.

5

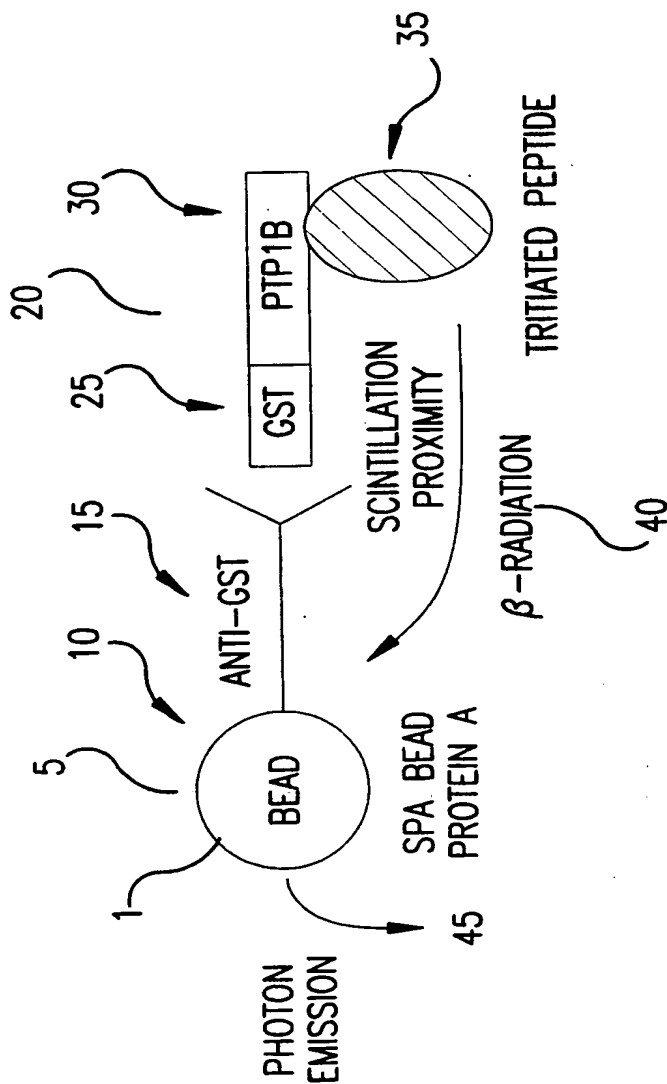


FIG.1

FIG. 2A

541	GACTTTGGAGTCCCTGAATCACCAGCCTCATTCTTGAACCTTCTTTTCAAAGTCCGAGAG	600
181	CTGAAACCTCAGGGACTTAGTGGTTCGGAGTAAGAACTTGAAAGAAAAGTTTCAGGCTCTC	200
	AspPheGlyValProGluSerProAlaSerPheLeuAsnPheLeuPheLysValArgGlu	
601	TCAGGGTCACTCAGCCCGGAGCACGGGCCCGTTGTGGTGCAGTGCAGGCATCGGC	660
201	AGTCCCAGTGAGTCGGGCCTCGTGCCCGGGCAACACCACGTGACGTACGTCCGTAGCCG	220
	SerGlySerLeuSerProGluHisGlyProValValValHisCysSerAlaGlyIleGly	
661	AGGTCTGGAACCTTCTGTCTGGCTGATACCTGCCTCCTGCTGATGGACAAGAGGAAAGAC	720
221	TCCAGACCTTGAAGACAGACCGACTATGGACGGAGGACGACTACCTGTTCTCCTTTCTG	240
	ArgSerGlyThrPheCysLeuAlaAspThrCysLeuLeuLeuMetAspLysArgLysAsp	
721	CCTTCTTCCGTTGATATCAAGAAAGTGCTGTTAGAAATGAGGAAGTTTCGGATGGGGTTG	780
241	GGAAGAAGGCAACTATAGTTCTTTACGACAATCTTTACTCCTTCAAAGCCTACCCCAAC	260
	ProSerSerValAspIleLysLysValLeuLeuGluMetArgLysPheArgMetGlyLeu	
781	ATCCAGACAGCCGACCAGCTGCGCTTCTCCTACCTGGCTGTGATCGAAGGTGCCAAATTC	840
261	TAGGTCTGTTCGGCTGGTCGACGCGAAGAGGATGGACCGACACTAGCTTCCACGGTTTAAG	
	IleGlnThrAlaAspGlnLeuArgPheSerTyrLeuAlaValIleGluGlyAlaLysPhe	
841	ATCATGGGGGACTCTTCCGTGCAGGATCAGTGGAAGGAGCTTTCCCACGAGGACCTGGAG	900
	TAGTACCCCTGAGAAGGCACGTCCTAGTCACCTTCCTCGAAAGGGTGCTCCTGGACCTC	
	IleMetGlyAspSerSerValGlnAspGlnTrpLysGluLeuSerHisGluAspLeuGlu	
901	CCCCACCCGAGCATATCCCCCACCTCCCCGGCCACCCAAACGAATCCTGGAGCCACACTGA	960
301	GGGGGTGGGCTCGTATAGGGGGGTGGAGGGGCCGGTGGGTTTGCTTAGGACCTCGGTGTGACT	320
	ProProProGluHisIleProProProProArgProProLysArgIleLeuGluProHisEnd	

FIG. 2B

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1 GAAACAAGCACTGGATTCCATATCCCACTGCCAAAACCGCATGGTTCAGATTATCGCTAT 60
-----+-----+-----+-----+-----+-----+
CTTTGTTTCGTGACCTAAGGTATAGGGTGACGGTTTTTGCGTACCAAGTCTAATAGCGATA

61 TGCAGCTTTTCATCATAATACACACCTTTGCTGCCGAAACGAAGCCAGACAACAGATTTC 120
-----+-----+-----+-----+-----+-----+
ACGTCGAAAGTAGTATTATGTGTGGAACGACGGCTTTGCTTCGGTCTGTTGTCTAAAGG

121 ATCAGCAGGATGTGGGGGCTCAAGGTTCTGCTGCTACCTGTGGTGAGCTTTGCTCTGTAC 180
-----+-----+-----+-----+-----+-----+
TAGTCGTCTACACCCCGAGTTCCAAGACGACGATGGACACCACTCGAAACGAGACATG
MetTrpGlyLeuLysValLeuLeuLeuProValValSerPheAlaLeuTyr

181 CCTGAGGAGATACTGGACACCCACTGGGAGCTATGGAAGAAGACCCACAGGAAGCAATAT 240
-----+-----+-----+-----+-----+-----+
GGACTCCTCTATGACCTGTGGGTGACCCTCGATACCTTCTTCTGGGTGTCCTTCGTTATA
ProGluGluIleLeuAspThrHisTrpGluLeuTrpLysLysThrHisArgLysGlnTyr

241 AACACAAGGTGGATGAAATCTCTCGGCGTTTAATTTGGGAAAAAACCTGAAGTATATT 300
-----+-----+-----+-----+-----+-----+
TTGTTGTTCCACCTACTTTAGAGAGCCGAAATTAACCCTTTTTTTGGACTTCATATAA
AsnAsnLysValAspGluIleSerArgArgLeuIleTrpGluLysAsnLeuLysTyrIle

301 TCCATCCATAACCTTGAGGCTTCTCTGGTGTCCATACATATGAACTGGCTATGAACCAC 360
-----+-----+-----+-----+-----+-----+
AGGTAGGTATTGGAACCTCGAAGAGAACCACAGGTATGTATACTTGACCGATACTTGGTG
SerIleHisAsnLeuGluAlaSerLeuGlyValHisThrTyrGluLeuAlaMetAsnHis

361 CTGGGGGACATGACCAGTGAAGAGGTGGTTCAGAAGATGACTGGACTCAAAGTACCCCTG 420
-----+-----+-----+-----+-----+-----+
GACCCCTGTACTGGTCACTTCTCCACCAAGTCTTCTACTGACCTGAGTTTCATGGGGAC
LeuGlyAspMetThrSerGluGluValValGlnLysMetThrGlyLeuLysValProLeu

421 TCTCATTCCCGCAGTAATGACACCCTTTATATCCCAGAATGGGAAGGTAGAGCCCCAGAC 480
-----+-----+-----+-----+-----+-----+
AGAGTAAGGGCGTCATTACTGTGGGAAATATAGGGTCTTACCCTTCCATCTCGGGGTCTG
SerHisSerArgSerAsnAspThrLeuTyrIleProGluTrpGluGlyArgAlaProAsp

481 TCTGTGCACTATCGAAAGAAAGGATATGTTACTCCTGTCAAAAATCAGGGTCAGTGTGGT 540
-----+-----+-----+-----+-----+-----+
AGACAGCTGATAGCTTTCTTTCTTATACAATGAGGACAGTTTTTAGTCCAGTCACACCA
SerValAspTyrArgLysLysGlyTyrValThrProValLysAsnGlnGlyGlnCysGly

FIG. 3A

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541 TCCTGTTGGGCTTTTAGCTCTGTGGGTGCCCTGGAGGGCCAACTCAAGAAGAAAAGTGGC 600
-----+-----+-----+-----+-----+-----+
AGGACAACCCGAAAATCGAGACACCCACGGGACCTCCCGGTTGAGTTCTTCTTTTGACCG
SerCysTrpAlaPheSerSerValGlyAlaLeuGluGlyGlnLeuLysLysLysThrGly
139

601 AACTCTTAAATCTGAGTCCCCAGAACCTAGTGGATTGTGTGTCTGAGAATGATGGCTGT 660
-----+-----+-----+-----+-----+-----+
TTTGAGAATTTAGACTCAGGGGTCTTGATCACCTAACACACAGACTCTTACTACCGACA
LysLeuLeuAsnLeuSerProGlnAsnLeuValAspCysValSerGluAsnAspGlyCys

661 GGAGGGGGCTACATGACCAATGCCTTCCAATATGTGCAGAAGAACCGGGGTATTGACTCT 720
-----+-----+-----+-----+-----+-----+
CCTCCCCCGATGTACTGGTTACGGAAGGTTATACACGTCTTCTTGCCCCATAACTGAGA
GlyGlyGlyTyrMetThrAsnAlaPheGlnTyrValGlnLysAsnArgGlyIleAspSer

721 GAAGATGCCTACCCATATGTGGGACAGGAAGAGAGTTGTATGTACAACCCAACAGGCAAG 780
-----+-----+-----+-----+-----+-----+
CTTCTACGGATGGGTATACACCCTGTCTTCTCTCAACATACATGTTGGGTGTCCGTTC
GluAspAlaTyrProTyrValGlyGlnGluGluSerCysMetTyrAsnProThrGlyLys

781 GCAGCTAAATGCAGAGGGTACAGAGAGATCCCCGAGGGGAATGAGAAAGCCCTGAAGAGG 840
-----+-----+-----+-----+-----+-----+
CGTCGATTTACGTCTCCCATGTCTCTCTAGGGGCTCCCCTTACTCTTTCGGGACTTCTCC
AlaAlaLysCysArgGlyTyrArgGluIleProGluGlyAsnGluLysAlaLeuLysArg

841 GCAGTGGCCCGAGTGGGACCTGTCTCTGTGGCCATTGATGCAAGCCTGACCTCCTTCCAG 900
-----+-----+-----+-----+-----+-----+
CGTCACCGGGCTCACCTGGACAGAGACACCGGTAACCTACGTTCCGACTGGAGGAAGGTC
AlaValAlaArgValGlyProValSerValAlaIleAspAlaSerLeuThrSerPheGln

901 TTTTACAGCAAAGGTGTGTATTATGATGAAAGCTGCAATAGCGATAATCTGAACCATGCG 960
-----+-----+-----+-----+-----+-----+
AAAATGTCGTTTCCACACATAATACTACTTTTCGACGTTATCGCTATTAGACTTGGTACGC
PheTyrSerLysGlyValTyrTyrAspGluSerCysAsnSerAspAsnLeuAsnHisAla

961 GTTTTGGCAGTGGGATATGGAATCCAGAAGGGAAACAAGCACTGGATAATTAACCAACAGC 1020
-----+-----+-----+-----+-----+-----+
CAAAACCGTCACCTATACCTTAGGTCTTCCCTTTGTTTCGTGACCTATTAATTTTGTGCG
ValLeuAlaValGlyTyrGlyIleGlnLysGlyAsnLysHisTrpIleIleLysAsnSer

1021 TGGGGAGAAAAGTGGGGAAACAAAGGATATATCCTCATGGCTCGAAATAAGAACAACGCC 1080
-----+-----+-----+-----+-----+-----+
ACCCCTCTTTTGACCCCTTTGTTTCCTATATAGGAGTACCGAGCTTTATTCTTGTGCGG
TrpGlyGluAsnTrpGlyAsnLysGlyTyrIleLeuMetAlaArgAsnLysAsnAsnAla

FIG.3B

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1081 TGTGGCATTGCCAACCTGGCCAGCTTCCCCAAGATGTGACTCCAGCCAGCCAAATCCATC 1140
-----+-----+-----+-----+-----+-----+
ACACCGTAACGGTTGGACCGGTCGAAGGGGTTCTACACTGAGGTCGGTCGGTTTAGGTAG
CysGlyIleAlaAsnLeuAlaSerPheProLysMetEnd

1141 CTGCTCTTCCATTTCTTCCACGATGGTGCA GTAACGATGCACTTTGGAAGGGAGTTGG 1200
-----+-----+-----+-----+-----+-----+
GACGAGAAGGTAAAGAAGGTGCTACCACGTCACATTGCTACGTGAAACCTTCCCTCAACC

1201 TGTGCTATTTTTGAAGCAGATGTGGTGATACTGAGATTGTCTGTT CAGTTTCCCCATTTG 1260
-----+-----+-----+-----+-----+-----+
ACACGATAAAAACTTCGTCTACACCACTATGACTCTAACAGACAAGTCAAAGGGGTAAAC

1261 TTTGTGCTTCAAATGATCCTTCCTACTTTGCTTCTCTCCACCCATGACCTTTTTCACTGT 1320
-----+-----+-----+-----+-----+-----+
AAACACGAAGTTTACTAGGAAGGATGAAACGAAGAGAGGTGGGTACTGGAAAAAGTGACA

1321 GGCCATCAGGACTTTCCCTGACAGCTGTGTACTCTTAGGCTAAGAGATGTGACTACAGCC 1380
-----+-----+-----+-----+-----+-----+
CCGGTAGTCCTGAAAGGGACTGTCGACACATGAGAATCCGATTCTCTACACTGATGTCGG

1381 TGCCCCCTGACTGTGTTGTCCCAGGGCTGATGCTGTACAGGTACAGGCTGGAGATTTTCAC 1440
-----+-----+-----+-----+-----+-----+
ACGGGGACTGACACAACAGGGTCCCGACTACGACATGTCCATGTCCGACCTCTAAAAGTG

1441 ATAGGTTAGATTCTCATTACGGGACTAGTTAGCTTTAAGCACCCCTAGAGGACTAGGGTA 1500
-----+-----+-----+-----+-----+-----+
TATCCAATCTAAGAGTAAGTGCCCTGATCAATCGAAATTCGTGGGATCTCCTGATCCCAT

1501 ATCTGACTTCTCACTTCCTAAGTTCCCTTCTATATCCTCAAGGTAGAAATGTCTATGTTT 1560
-----+-----+-----+-----+-----+-----+
TAGACTGAAGAGTGAAGGATTCAAGGGAAGATATAGGAGTTCCATCTTTACAGATACAAA

1561 TCTACTCCAATTCATAAATCTATTCATAAGTCTTTGGTACAAGTTTACATGATAAAAAGA 1620
-----+-----+-----+-----+-----+-----+
AGATGAGGTTAAGTATTTAGATAAGTATT CAGAAACCATGTTCAAATGTACTATTTTTCT

1621 AATGTGATTTGTCTTCCCTTCTTTGCACTTTTGAAATAAAGTATTTATC 1669
-----+-----+-----+-----+-----+
TTACACTAAACAGAAGGGAAGAAACGTGAAAAC TTTATTTTCATAAATAG

FIG. 3C

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1	CTGCAGGAATTCGGCACGAGGGGTGCTATTGTGAGGCGTTGTAGAAGTTAATAAAGGTA -----+-----+-----+-----+-----+-----+-----+ GACGTCCTTAAGCCGTGCTCCCCACGATAAACTCCGCCAACATCTTCAATTATTTCCAT	60
61	TCCATGGAGAACACTGAAAACCTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCAAAG -----+-----+-----+-----+-----+-----+-----+ AGGTACCTCTTGTGACTTTTGAGTCACCTAAGTTTTAGGTAATTTTTAAACCTTGGTTTC MetGluAsnThrGluAsnSerValAspSerLysSerIleLysAsnLeuGluProLys	120
121	ATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGTTATAAAATG -----+-----+-----+-----+-----+-----+-----+ TAGTATGTACCTTCGCTTAGTTACCTGAGACCTTATAGGGACCTGTTGTCAATATTTTAC IleIleHisGlySerGluSerMetAspSerGlyIleSerLeuAspAsnSerTyrLysMet	180
181	GATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAATTTTCATAAGAGCACT -----+-----+-----+-----+-----+-----+-----+ CTAATAGGACTCTACCCAAATACATATTATTAATTATTATTCTTAAAAGTATTCTCGTGA AspTyrProGluMetGlyLeuCysIleIleIleAsnAsnLysAsnPheHisLysSerThr	240
241	GGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCAAACCTCAGGGAAACATTCAGA -----+-----+-----+-----+-----+-----+-----+ CCTTACTGTAGAGCCAGACCATGTCTACAGCTACGTCGTTTGGAGTCCCTTTGTAAGTCT GlyMetThrSerArgSerGlyThrAspValAspAlaAlaAsnLeuArgGluThrPheArg	300
301	AACTTGAAATATGAAGTCAGGAATAAAAATGATCTTACACGTGAAGAAATTGTGGAATTG -----+-----+-----+-----+-----+-----+-----+ TTGAACTTTATACTTCAGTCCTTATTTTTACTAGAATGTGCACTTCTTTAACACCTTAAC AsnLeuLysTyrGluValArgAsnLysAsnAspLeuThrArgGluGluIleValGluLeu	360
361	ATGCGTGATGTTTCTAAAGAAGATCACAGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTG -----+-----+-----+-----+-----+-----+-----+ TACGCACTACAAAGATTTCTTCTAGTGTGTTTTCTCGTCAAAACAAACACACGAAGAC MetArgAspValSerLysGluAspHisSerLysArgSerSerPheValCysValLeuLeu	420
421	AGCCATGGTGAAGAAGGAATAATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAATA -----+-----+-----+-----+-----+-----+-----+ TCGGTACCACTTCTCCTTATTA AAAACCTTGTTTACCTGGACAACCTGGACTTTTTTTAT SerHisGlyGluGluGlyIleIlePheGlyThrAsnGlyProValAspLeuLysLysIle	480
481	ACAACTTTTTTCAGAGGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAACTTTTCATT -----+-----+-----+-----+-----+-----+-----+ TGTTTGAAAAGTCTCCCTAGCAACATCTTCAGATTGACCTTTTGGGTTTGAAAAGTAA ThrAsnPhePheArgGlyAspArgCysArgSerLeuThrGlyLysProLysLeuPheIle	540

FIG.4A

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541 ATTCAGGCCTGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTGAT 600
 -----+-----+-----+-----+-----+-----+
 TAAGTCCGGACGGCACCATGTCTTGACCTGACACCGTAACTCTGTCTGTACCACAATA
 IleGlnAlaCysArgGlyThrGluLeuAspCysGlyIleGluThrAspSerGlyValAsp
 163

601 GATGACATGGCGTGCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCCACA 660
 -----+-----+-----+-----+-----+-----+
 CTAAGTGTACCGCACAGTATTTTATGGTCACCTCCGGCTGAAGAACATACGTATGAGGTGT
 AspAspMetAlaCysHisLysIleProValGluAlaAspPheLeuTyrAlaTyrSerThr

661 GCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATCCAGTCGCTT 720
 -----+-----+-----+-----+-----+-----+
 CGTGGACCAATAATAAGAACCGCTTTAAGTTTCCTACCGAGGACCAAGTAGGTCAGCGAA
 AlaProGlyTyrTyrSerTrpArgAsnSerLysAspGlySerTrpPheIleGlnSerLeu

721 TGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCACATTCTTACCCGGGTT 780
 -----+-----+-----+-----+-----+-----+
 ACACGGTACGACTTTGTCTACGGCTGTTTCGAACTTAAATACGTGTAAGAATGGGCCCAA
 CysAlaMetLeuLysGlnTyrAlaAspLysLeuGluPheMetHisIleLeuThrArgVal

781 AACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTTGACGCTACTTTTCATGCAAAG 840
 -----+-----+-----+-----+-----+-----+
 TTGGCTTTCCACCGTTGTCTTAACTCAGGAAAAGGAACTGCGATGAAAAGTACGTTTC
 AsnArgLysValAlaThrGluPheGluSerPheSerPheAspAlaThrPheHisAlaLys

841 AAACAGATTCCATGTATTGTTTCCATGCTCACAAAAGAACTCTATTTTATCACTAAAGA 900
 -----+-----+-----+-----+-----+-----+
 TTTGTCTAAGGTACATAACAAAGGTACGAGTGTCTTCTTGAGATAAAAATAGTGATTTCT
 LysGlnIleProCysIleValSerMetLeuThrLysGluLeuTyrPheTyrHisEnd

901 AATGGTTGGTTGGTGGTTTTTTTTAGTTTGTATGCCAAGTGAGAAGATGGTATATTTGGT 960
 -----+-----+-----+-----+-----+-----+
 TTACCAACCAACCACCAAAAAAATCAAACATACGGTTCCTCTTCTACCATATAAACCA

961 ACTGTATTTCCCTCTCATTTTGACCTACTCTCATGCTGCAG 1001
 -----+-----+-----+-----+-----+
 TGACATAAAGGGAGAGTAAACTGGATGAGAGTACGACGTC

FIG.4B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 97/00824

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K5/08 G01N33/573

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R L WANGE ET AL.: "F2(PMP)2-TAMzeta3, a novel competitive inhibitor of the binding of ZAP-70 to the T cell antigen receptor, blocks early cell signaling" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 370, no. 2, 13 January 1995, MD US, pages 944-948, XP002056490 see the whole document --- -/--	1,2



Further documents are listed in the continuation of box C.



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* Special categories of cited documents:

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Date of the actual completion of the international search

20 February 1998

Date of mailing of the international search report

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Masturzo, P

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 97/00824

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S R EATON ET AL.: "Structure-activity relationship of peptides that block the association of PDGF beta-trceptor with phosphatidylinositol 3-kinase" PEPTIDES. CHEMISTRY, STRUCTURE AND BIOLOGY. PROCEEDINGS OF THE 14TH AMERICAN PEPTIDE SYMPOSIUM, JUNE 18-23, 1995, COLUMBUS, OHIO, USA, 1996, MAYFLOWER SCIENTIFIC LTD, ENGLAND, pages 414-415, XP002056491 see the whole document ---	1,2
A	M F GORDEEV ET AL.: "N-alpha-FMOC-4-phosphono (difluoromethyl)-L-phenylalanine: a new O-phosphotyrosine isosteric building block suitable for direct incorporation into peptides " TETRAHEDRON LETTERS., vol. 35, no. 41, 10 October 1994, OXFORD GB, pages 7585-7588, XP000616320 see the whole document ---	1,2
X,P	WO 97 08300 A (ARIAD PHARMACEUTICALS, INC.) 6 March 1997 see page 17, compound 9 -----	1,2

Information on patent family members

PCT/CA 97/00824

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